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Pennsylvania—1991

Environmental Assessment and Finding of No Significant Impact

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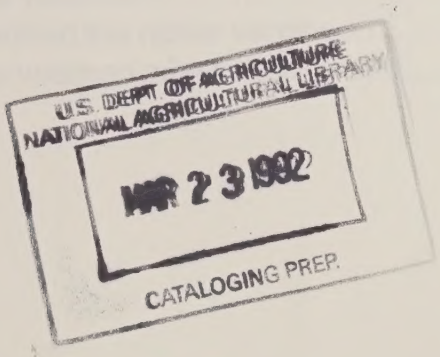

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I. Abstract

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has received a request under the Virus-Serum-Toxin Act (21 U.S.C. 151 *et seq.)* from the Wistar Institute of Anatomy and Biology (Wistar) to conduct a field trial with a live experimental vaccinia-vector rabies viral vaccine for raccoons, designated by Wistar as V-RG. The field trial will be conducted on state-owned land in Sullivan County, Pennsylvania. In developing the vaccine, genetic engineering procedures were employed to insert a rabies virus coat glycoprotein gene into the vaccinia virus genome. The foreign gene encodes the rabies viral glycoprotein responsible for induction of rabies virus neutralizing antibody (VNA). The insertion inactivated the vaccinia virus thymidine kinase (TK) gene, which is implicated in vaccinia virus virulence. This vaccine is capable of eliciting an immune response that protects raccoons from virulent street rabies virus infection without causing the disease. The V-RG vaccine was shown to be safe in animals in a variety of safety and efficacy studies that were carried out in laboratory isolation.

Field trials of the V-RG vaccine in Belgium and France during 1987-91 have so far demonstrated an absence of adverse effects in human and wildlife populations. An island field trial of this vaccine was authorized by USDA and initiated in Virginia by Wistar in 1990. Initial results have so far demonstrated vaccine-bait uptake of over 70%, specificity for the raccoon target animal, and no evidence of either vaccinia or rabies disease in raccoons or nontarget animals. Monitoring of personnel involved in the field trial for evidence of exposure to these viruses has also been negative.

An analysis of the environmental consequences of a proposed field trial of the V-RG vaccine in Sullivan County, Pennsylvania, indicates that no environmental impacts are anticipated.

The issuance of authorization to conduct this field trial permits the developer to compile additional data for vaccine safety and other requirements that are a prerequisite to additional field trials and potential licensing of the live virus vaccine. This trial will be conducted with state approval, in accordance with requirements published in Title 9, Section 103.3 of the Code of Federal Regulations.

II. Finding Of No Significant Impact

The Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture (USDA) has prepared an Environmental Assessment (EA) that presents a discussion of scientific data and other supportive information which is being considered by APHIS prior to permitting a limited field trial in Pennsylvania of a vaccinia-vector recombinant rabies vaccine (V-RG) for raccoons. The EA describes the proposed field trial, provides background information on rabies and vaccinia viruses, discusses the purpose and need for the field trial, describes various characteristics of the specific trial site, and considers the environmental consequences of the trial. Mitigations to minimize potential impacts on the environment are examined, as is a monitoring plan. Based on this analysis, APHIS has tentatively determined that permitting the proposed field trial with the V-RG vaccine would not have a significant impact on the quality of the human environment. Such a preliminary finding is supported by the following:

1. Genetic engineering procedures were employed to incorporate only the rabies glycoprotein gene within the thymidine kinase (TK) locus of vaccinia virus. The resultant vaccinia virus cannot induce rabies.

2. The V-RG virus has been shown to cause no adverse clinical signs or gross or histopathological lesions, yet is fully capable of eliciting an immune response that protects a variety of species from virulent rabies virus challenge. The V-RG virus is unable to evoke antibodies to other rabies viral structural proteins.

3. The TK gene insertion is a stable characteristic of the V-RG virus with a very low probability of loss or reversion.

4. The V-RG virus does not contain an oncogene or cancer-causing substance, and does not contain any new genetic information to enhance the likelihood of it becoming oncogenic.

5. Biological transmission of the V-RG virus could not be demonstrated in laboratory studies involving more than 40 species of domesticated and wild mammals and birds.

6. In rare instances, contact (mechanical) transmission between animals was observed immediately after oral administration of the V-RG vaccine. No adverse effects from these transmissions were observed, and no adverse outcomes are expected from similar exposures during the field trial.

7. Laboratory containment experiments demonstrate that the V-RG vaccine is non-pathogenic, safe, and efficacious in a variety of laboratory animal model systems and a number of target and non-target species, to include the major terrestrial wildlife and domestic animal reservoirs of rabies.

8. Previous field trials of the V-RG vaccine in Europe and Virginia have not demonstrated adverse effects of V-RG for workers, local human populations, or wildlife.

9. In the proposed field trial, mitigations will be in place to minimize the potential impact of the V-RG vaccine and the field trial itself on humans and the environment. These measures will include immunization of project workers and restricted access to the trial site during the 2-week initial period of vaccine-bait placement.

10. Monitoring of wildlife and human populations in the area of the field trial will continue for 1 year after initiation of the trial.

James W. Glosser, Administrator

6/7/91

Date

III. Introduction

A. Proposed Action

The Wistar Institute of Anatomy and Biology (Wistar) has requested authorization from APHIS to conduct a field test of a genetically engineered recombinant live vaccinia virus vector rabies vaccine (designated by Wistar as V-RG) under the provisions of 9 CFR 103.3. The experimental vaccine to be tested has been genetically derived by recombinant DNA techniques. The experimental recombinant live vaccinia virus vaccine contains the glycoprotein gene from an attenuated rabies virus strain (ERA). Cells that are infected with the vaccine virus produce the rabies glycoprotein, which elicites production of rabies neutralizing antibodies. Inoculation with or ingestion of V-RG is thus capable of conferring protective immunity against rabies.

1. Applicant

The Wistar Institute, founded in 1892 and located in Philadelphia, PA, is a major biomedical research organization with an international reputation in human and wildlife rabies vaccine development. The post-exposure human diploid cell rabies vaccine (HDCV) that is now used worldwide was developed by Wistar, as was a veterinary rabies vaccine.

2. Objectives

The specific objective of this proposed trial is to collect information on the safety and efficacy of the V-RG experimental vaccine under field conditions. The vaccine was developed to reduce transmission of rabies within wildlife populations and from wildlife to domestic animals or humans. In this field test, the vaccine will be offered by the oral route to raccoons of all ages to determine the vaccine's safety and immunogenicity under field conditions. The vehicle for vaccine delivery (bait) will also contain biomarkers as a means of monitoring exposure rates to the vaccine. Both target and nontarget animals will be monitored to determine possible infection and transmission of the vaccinia virus used as a vector in the vaccine. Concurrent studies will also examine vaccine stability under field conditions. Wild-caught raccoons from the study populations will subsequently be challenged in Wistar containment facilities with a local strain of street rabies virus to assess the level of immunity (efficacy). More fundamental objectives of this and other field trials of the V-RG vaccine are to demonstrate the feasibility of the vaccine construction, the effectiveness of the delivery system, and the absence of environmental hazards. The results of this study will be prepared for formal publication in a refereed scientific journal, and should confirm the safety of the oral rabies vaccine in the field and its efficacy in laboratory challenge studies. Such success may lead to a proposal for large-scale application of the vaccine and bait to selected rabies epizootic areas within the mid-Atlantic region, as a means of further demonstrating safety and efficacy in the field under various ecological conditions.

3. Field Trial

a. Site Selection Criteria

The Wistar Institute's criteria for the selection of suitable study sites included: a relatively large and stable target raccoon population; very low incidence (or absence) of rabies in local terrestrial mammals; restricted access by the public; limited area (e.g., 500-1000 ha) for logistical considerations; lack of known endangered or threatened species; topographical features that would decrease the probability of wildlife dispersal; diverse habitat and nontarget wildlife populations that would represent the surrounding area; recent background data on test site biotic composition in general, and raccoon history and population trends specifically; and cooperation by local authorities and surrounding community.

b. Location

A field trial site was selected on Pennsylvania State Game Land (SGL) #13, Sullivan County (76° 20'W, 41° 18'N), and adjacent Lycoming County, after consultation with the Pennsylvania (PA) Game Commission and the PA Departments of Health and Agriculture (see map, fig. 1). The general study area consists of 20,051 ha (49,527 acres) of woodland with several stream valleys. Two stream valley areas will be used for vaccine-bait placement; along Shingle Mill Road west to the junction with Hemlock Run Road, and along Grassy Hollow Road from Lewis Falls to Ridge Road. These sites are separated from each other by 7 km, are at least 1 to 2 km from the periphery of the SGL, and have a combined area of approximately 1000 ha. Surveillance areas comprising approximately 2000 ha will be established adjacent to the vaccine-bait areas (see section V.A. for a more detailed site description).

c. Protocol

(1) Off-site preparation of vaccine and bait

Primary assembly of the baits and their loading with vaccine will be conducted under Category 2 biocontainment conditions at the Wistar Institute in Philadelphia, PA. V-RG virus will be removed from storage and titrated to determine the number of $TCID_{50}$ per unit volume (see appendix A for details of V-RG preparation). At the same time, an aliquot of the V-RG solution will be sent to the National Veterinary Services Laboratory (USDA-APHIS) in Ames, IA, for confirmation of purity and concentration. Approximately 1.0 ml of theV-RG virus (10^8 pfu/ml) will be placed in each of several thousand wax ampules. These sealed vaccine chambers will be then each placed inside a fishmeal-polymer cylinder bait developed and field-tested by Wistar. Fishmeal-polymer plugs will be inserted into each end of the cylinder and sealed with paraffin. The vaccine-bait units will be secured in water-tight and sealed drums and refrigerated at Wistar prior to delivery. The assembled fishmeal-polymer baits with vaccine will then be transported in trucks under refrigeration to the field site by Wistar personnel. Labeling

and packaging will conform to current U.S. Public Health Service recommendations for handling, storage, and transportation of etiological agents.

(2) On-site preparation of vaccine-bait combination

Each vaccine-bait unit will be placed in a polyethylene bag stamped with a written warning and identification label. Approximately 50 ml of a slurry containing water, sugar, egg, vegetable oil, and fermented fish/shellfish will be added to the bag to enhance bait attractiveness to raccoons and repellency to humans. A seromarker, 250 mg of sulfadimethoxine (SDM), also will be added to the slurry.

(3) Vaccine-bait placement

Single vaccine-bait units will be hand-placed at approximately 5,000 uniquely numbered flagged sites along predesignated transects reflecting sites of suggested raccoon activity (dens and foraging areas) in the study area to achieve an approximate baiting density of at least 5 baits/ha (2 baits/acre). On a single hectare each in the Shingle Mill and Grassy Hollow study sites, bait density will be increased to approximately 100 baits/ha (40 baits/acre), to maximize the likelihood of contact by nontarget (e.g., small mammal) species.

(4) Subsequent field activity

(a) Baits

Vaccine-bait units will be checked each morning for the 10 days after placement, with written records maintained for each unit and a photographic or videotape record obtained for selected units with evidence of animal contact. Intact or fragmented vaccine chambers and surrounding debris will also be collected on the 10th day and tested for virus activity. Any remaining field baits, bags, and associated debris on day 10 will be transported to Wistar for proper disposal.

Tracking pits will be randomly established at approximately 150 of the bait stations. These stations will consist of a cleared and level 1 m^2 plot filled with an impressionable substrate and with the bait-vaccine unit in the center.

Vaccine-bait units in bags will be placed in screened enclosures in full sunlight, shade, and intermediate (sun/shade) habitat areas to monitor the stability of the vaccine over time under environmental conditions and protected from potential consumers. Samples will be removed daily through day 14 and tested in the laboratory for virus activity.

All animal handling will be conducted in an appropriate manner, with all procedures previously approved by the Wistar Institute Animal Care and Use Committee, a federally mandated mechanism (9 CFR 2.31) for review of all protocols requiring use of animals.

(b) Animal trapping

General. Vertebrate populations will be sampled by live-trapping, snap-trapping (lethal), and shooting. For the first 14 days after vaccine placement, traps will be set in the evening and checked and reset the following morning; the minimum trapping effort during this period will be 500 trap-nights. For the long-term surveillance period beginning on day 14 and continuing for approximately the next 11 months, traps will be set for 4- to 5-day periods twice a month; the minimum effort during this period will be 400 trap-nights per month. Live trapping will be supplemented when appropriate with shooting to increase the sample size of nontarget species.

Live animals will be sedated with a mixture of ketamine (10mg/kg) and xylazine (0.4 mg/kg) administered intramuscularly prior to handling. All animals will be identified to species, and a blood sample and oral and anal swabs obtained. A subsequent physical examination will record sex, reproductive condition, relative age, and weight. If released, animals will be identified by a unique ear-tag number and allowed to recover from anaesthesia at the point of capture. If collected for necropsy, live-trapped animals will be euthanized by cervical dislocation (rodents) or by intravenous barbiturate overdose. Sera from live-trapped animals for the first 7 days after vaccine placement will be tested for the presence of the biomarker sulfadimethoxine to determine bait contact.

Animals killed in traps or shot will be processed in the same manner as live animals whenever possible, with subsequent retention for post-mortem examination, virus isolation, and biomarker determination. All observed fresh field carcasses of any species will be collected for post-mortem examination, as will any trapped animal exhibiting abnormal clinical signs or gross lesions.

Raccoons. Most of the general live-trapping effort will be directed toward the raccoon to determine contact and response to the vaccine and associated health and movement. An effort will be made during the summer to capture pregnant and lactating females and females with young for examination following vaccine deployment. During the first 2 weeks after vaccine placement, two raccoons every other day will be captured and euthanized (maximum of 14 raccoons) for post-mortem examination, virus isolation, and antibody determination. In the following 11 months, two raccoons each month will also be euthanized and processed in a similar manner. One-half of the collected raccoons will be obtained from the vaccine-bait placement areas, the other half from the surveillance areas.

Nontarget animals. During the period of bait placement and subsequent surveillance, as many as 30 individuals of several species of small mammals will be captured, examined, and released (subject to local densities and catchability), to include meadow vole (*Microtus pennsylvanicus*), Norway rat (*Rattus norvegicus*), cottontail rabbit (*Sylvilagus floridanus*), and species of field mice (*Peromyscus* spp.). Smaller numbers of carnivores, such as red fox (*Vulpes vulpes*), opossum (*Didelphis virginianus*), and skunk (*Mephitis*

mephitis), and other nontarget species such as white-tailed deer (Odocoileus virginianus), will also be captured, examined, and released. Limited numbers of selected nontarget species will be collected for biomarker determinations, serology, virus isolation, post-mortem analysis, and histopathological examination.

(c) Adjacent areas

Animal collections will also be conducted over a 12-month period on sites adjacent to the vaccine placement area. These surveillance areas are to the immediate north and south, with intervening elevated ridges. Animals from these areas will be live-trapped and examined for possible movement from the vaccine area (based on ear-tag identification), and for prevalence of biomarker and rabies antibody. A minimum effort of 100 trap-nights per month will be expended on these adjacent surveillance areas.

(d) Radiotelemetry

Radiotelemetry of raccoons will be used to monitor general activity levels, habitat use, and the ranges of approximately 20 to 30 raccoons on the study sites. After capture, radio collars will be fitted to the neck of adult raccoons. These telemetry units have both activity and mortality modes, permitting recording of location and degree of activity. The inactivity associated with death can be detected rapidly, allowing recovery of such raccoons for immediate post-mortem examination. Pregnant and lactating females will be monitored intensively, and when possible associated progeny will be retrieved for examination and collection of blood samples.

(5) Subsequent lab activity

(a) Detection of biomarker, virus, and antibody

The biomarker placed in the polymer bait during placebo bait trials was a tetracycline (100 mg Panmycin) that is commonly used as a hard tissue (bone and teeth) marker and therefore can function as a post-mortem biomarker. Mandibular bone and teeth samples were collected from hunter-killed carcasses and animals live-trapped and sacrificed for background histopathology. Sections of the bone samples (10 micron) were mounted on microscope slides and examined under ultraviolet illumination for tetracycline deposition within cementum and dentine. The biomarker placed in the bait slurry will be sulfadimethoxine (SDM, 250 mg Albon R), a veterinary pharmaceutical used in the treatment of domestic animal gastrointestinal disorders. A commercial card test is available for rapid detection of SDM in blood sera, facilitating the use of SDM as a short-term biomarker.

Blood collected from animals in the field will be allowed to clot, and the serum removed and frozen at -20°C. In the laboratory, a modification (114) of the Rapid Fluorescent Focus Inhibition Test (RFFIT) will be used to determine the presence of rabies and other virus neutralizing antibodies.

Routine post-mortem pathological and parasitological exams will be conducted to determine the general health of captured animals (111). Detection of viruses will be by standard methods (109). If V-RG virus is isolated

from any tissue, it will be titrated and antigenically compared to the original V-RG virus vaccine using rabies glycoprotein-specific monoclonal antibodies (Mabs). Isolated viruses will be quantified and examined for changes in virulence through inoculation of laboratory animals, as compared to the original V-RG vaccine. Their TK status will also be determined.

(b) Efficacy test procedure

To assess vaccine efficacy, 12 months after vaccine deployment approximately 20 raccoons from the vaccine bait area will be collected and challenged at Wistar with rabies virus.

Theoretically, depending upon the interrelationship of natural background prevalence of rabies VNA, the adequacy of the biomarker, and the potential shedding ability of the V-RG virus, four different groups could exist in the raccoon population after vaccine deployment: (a) biomarker positive, rabies sero-positive; (b) biomarker negative, rabies sero-negative; (c) biomarker positive, rabies sero-negative; and (d) biomarker negative, rabies sero-positive. A maximum of 5 members of each potential group will be trapped from vaccine-bait and surveillance areas and transported to the Wistar laboratory. Following a 7 to 10 day adjustment period of antiparasite (e.g., arthropod, helminth) treatment, raccoons will be bled for rabies-specific VNA and challenged intramuscularly (i.m.) with a virulent dose of a local strain of street rabies virus. Following the challenge, animals will be examined daily for a minimum of 90 days for clinical signs attributable to rabies and then euthanized. Brain material from all raccoons, including rabies suspects, will be examined for rabies virus by the direct fluorescent antibody test. A final report will be submitted to APHIS officials at this time.

B. Background - Rabies

1. History and Characterization

Rabies is an acute infectious disease of the central nervous system (CNS). It is one of the oldest known diseases and is world wide in distribution, except for many islands (e.g., Pacific Oceania) or countries achieving secondary eradication (e.g., Great Britain) (122). All warm-blooded vertebrates are susceptible to the rabies virus, but in greatly varying degrees. The etiological agent is a member of the Rhabdoviridae, a family currently composed of some 80 described species of rod-shaped or bacilliform plant and animal viruses (81). Virion dimensions vary from 130-380 x 50-95 nm. The viral genome is single-stranded, negative-sense, nonsegmented RNA complexed with three proteins in a helical array, and surrounded by a lipid bilayer envelope in which the viral glycoprotein molecules are anchored (149).

Rabies virus is the type species of the *Lyssavirus* genus, which includes therabies-related viruses (Duvenhage, Lagos bat, Mokola, Kotokan, and Obodhiang) isolated from mammals or insects from the Old World. An unofficial subdivision of rabies viruses, which has been in vogue since the time of Pasteur, operationally separates "street" rabies viruses that exist in nature from "fixed" rabies viruses. Fixed viruses are field isolates that have

been secondarily adapted in the laboratory and which possess relatively predictable and stable biological attributes. For example, Pasteur's original field isolate of rabies from a cow was serially propagated in rabbits by intracerebral (i.c.) inoculation, so that with succeeding passages the time period between rabbit infection and death decreased from an average of 15 days to a "fixed" period of approximately 7 days (139). Since the 1880s, additional fixed rabies viruses have been developed (e.g., Flury HEP/LEP, Kelev, CVS, SAD, ERA, etc.) by secondary adaptation to growth in laboratory animals or cell culture, and have formed the basis of vaccine technology and rabies virus research to the present (139).

The domestic dog (*Canis familiaris*) is the principal host and major vector of rabies throughout the world (39). Rabies in dogs accounted for more than 85% of the 14,049 reported cases in Mexico in 1987 (24). Worldwide, rabies of dog origin accounts for the majority of the 50,000 to 70,000 human rabies deaths per year and for the more than 500,000 reported human post-exposure treatments. These cases are largely restricted to the equatorial regions of Asia, Latin America, and Africa. Most health authorities agree that the international reporting of human and animal rabies cases grossly underestimates the magnitude of the problem (39).

The predominant global wildlife rabies reservoirs belong to the Carnivora (58) and include arctic fox (*Alopex lagopus*) in the arctic areas; red fox (*Vulpes vulpes*) in eastern Canada, upper New York State, central and western Europe; gray fox (*Urocyon cinereoargenteus*) in scattered foci throughout the United States; skunks (*Mephitis mephitis* and *Spilogale putorius*) primarily in the midwestern U.S. and portions of western Canada; raccoon (*Procyon lotor*) in the southeastern and mid-Atlantic region of the U.S.; raccoon dog (*Nyctereutes procyanoides*) in eastern Europe and Scandinavia; jackals (*Canis* sp.) and other wild canids and viverrids (e.g., the yellow mongoose, *Cynictis penicillata*) in Asia and Africa. In the Indian mongoose (*Herpestes auropunctatus*), which was introduced into several Caribbean Islands for rat control during the 19th century, rabies persists as a major agricultural and public health threat. "Bat" rabies predominates as a New World phenomenon, described primarily in insectivorous species in the United States and Canada (some 40 species), and the three blood-eating vampire species (principally *Desmodus rotundus*) from Northern Mexico to Argentina. Ongoing epidemiological investigations should clarify the role of insectivorous bats in recently described cases of rabies in Europe. Rodent rabies is uniformly rare around the globe.

In contrast to the developing world, the distribution of the 4,808 reported animal rabies cases in the United States during 1989 demonstrates the disproportionate importance of a few wild mammalian species that serve as rabies reservoirs for the majority of other wildlife, domestic animals, and humans (105). For example, 88% of the cases reported in 1989 were from skunks, raccoons, foxes, and bats, while 12% were from domestic animals (54). Although skunks continue to be the most commonly reported rabid wild animal in the United States, the intensity of the mid-Atlantic raccoon

rabies epizootic is reflected in a 296% (481 to 1,906) increase in reported rabid raccoons from 1981 to 1983 (105). Also in contrast to the developing world, there are now fewer than two reported human fatalities per year on the average in the United states attributable to rabies (105). Despite the lack of appreciable human mortality in the United States, approximately 20,000 individuals receive rabies post-exposure treatments in this country annually.

2. Etiology

a. Pathogenesis

A generalized scheme for the pathogenesis of rabies virus infection is largely based upon experimental animal inoculation (87). Transmission usually originates from the bite of an infected animal, with the introduction of virus-laden saliva deep into the wound. Virus enters severed or intact unmyelinated peripheral nerves (e.g., a motor end-plate or the neuromuscular spindle apparatus). Virus may also enter by attachment to specific receptors (120). Rabies virus travels via peripheral nerves to the CNS, where replication and assembly of mature virions ensues. Movement across neuronal synapses may involve budding from plasma membranes, or spread may occur by direct cell-to-cell passage. Viremia has not been implicated to any significant extent. Successive cycles of replication and passive transport result in widespread dissemination throughout the brain. Infection progresses from the CNS, with the outward migration of virus through the nerve terminals of a variety of organs (most notably the salivary glands) via neural innervation of secretory cells. At the major portal of exit, the salivary glands, additional viral replication occurs. Variations on the above generalized scheme may include: local infection of rabies virus in striated muscle tissue at the bite site (25); infection of sensory neurons, such as olfactory neuroepithelia; aerosol transmission of virus under unique environmental conditions as found in a few bat caves (27); viral entry via the gastrointestinal route (103); sequestration of virus in additional non-nervous tissue (e.g., brown fat) (126); and rarely, transplacental transmission to offspring (53). The bite route is regarded as the primary means of rabies transmission.

The outcome of rabies virus-induced encephalitis is invariably death from neurological dysfunction of vital brain regions. Gross lesions are generally lacking. The histopathological lesions are nonspecific (e.g., neuronal degeneration, neuronophagia, and monocytic cell infiltration with perivascular cuffing), as would be expected from any viral inflammation of the CNS. In the past, cytoplasmic inclusion bodies of viral nucleocapsid (NC) aggregates, or Negri bodies, in CNS neurons formed the basis of post-mortem laboratory examination (in concert with i.c. inoculation of mice with suspected brain material). This method has been replaced by the more sensitive fluorescent antibody (FA) technique as the preferred method of rabies diagnosis (61).

Rabies virus infection is not synonymous with mortality. The outcome of infection is dependent upon the virus dose, the strain, the route of infection, the host species in question, and the age, physiological, and immunological

status of the host. The natural incubation period ranges from weeks to months, but is highly variable and dependent upon the same host-virus factors. The role played by humoral and cell-mediated immunity in the pathogenesis of rabies, and in viral evasion of such host-derived responses until late in the course of infection, is poorly understood.

Clinical signs in an affected animal usually appear concurrently with the presence of infectious virus in the saliva. Typical behavioral syndromes range from enhanced aggressiveness ("furious rabies") to apparent friendliness and loss of fear of humans, varying degrees of incoordination, convulsions, and ascending paralysis ("dumb rabies"). The clinical signs may be as subtle as inappetence or an alteration in vocalization, depending upon the degree of neocortical, limbic, or brainstem involvement.

b. Antigenic variation

The rabies glycoprotein, the outer viral surface protein, is the only primary viral subunit responsible for the induction of rabies virus neutralizing antibody (VNA); it also induces the stimulation of cytotoxic-T lymphocytes (150) and is probably involved in viral attachment to cellular receptors (120). It was by the application of monoclonal antibodies (Mabs) (62,102) to the study of the *Lyssavirus* genus (140) (previously felt to consist of a relatively homogeneous group of viruses) that considerable antigenic variation was demonstrated (41,42) among both fixed and street rabies viruses, and between rabies and the rabies-related viruses (141). Such Mabs have been particularly useful in determining the extent of natural antigenic variation among rabies viruses isolated from a variety of wildlife reservoirs within a fairly restricted geographical area. When interpreted under past and current epidemiological criteria, distinct species-associated patterns emerge (26,64, 113,119,127). In particular, distinctions are obvious between rabies viruses isolated from bats and terrestrial carnivores. Moreover, Mabs are a useful epidemiological tool to determine the source of rabies exposure to humans or domestic animals when a history of definitive contact is lacking (108).

3. Control

Efforts to control sylvatic (wild animal) rabies have involved: direct reduction of wildlife reservoir populations; establishment of an "immune barrier" between wildlife vectors and domestic species via mandatory vaccination of dogs and sometimes cats, and leash laws; and more recently, attempts to immunize reservoir wildlife populations. Long-lasting and widespread control of wildlife rabies appears to be attainable only through field immunization of primary sylvatic vectors (137). Progress in wildlife rabies immunization in the United States has been limited by several factors: vaccine availability; accessibility of individual wild animals; methods of vaccine delivery; and economic, safety, and sociopolitical considerations.

a. Reduction of Vector Population

Past attempts to control sylvatic rabies in wild carnivores were directed toward population reduction and removal of infected animals to reduce intraspecies transmission (69,70). The advantages of this technique are (1) no new biological introductions; (2) use of well-established techniques that can be integrated with sport hunting and trapping and can be administered through state wildlife agencies; and (3) direction to specific target species. Disadvantages include (1) high cost (69); (2) labor-intensiveness; (3) poor efficacy, since nonrabid animals will be removed as well as rabid or incubating animals; (4) lack of effect on human safety; (5) depending upon intensity of any given technique, potentially severe impact on environment, target, and nontarget domestic species; and (6) ethical considerations, especially with poisoning or gassing campaigns.

b. Non-Oral Vaccination

Several attempts have been made to live-trap and hand-vaccinate wildlife against rabies using an inactivated rabies virus as a vaccine (3,78,106). This practice attempts to create "immune barriers" to rabies between humans and domestic animals using inactivated virus vaccines and manual vaccination of target wildlife species (3,78). Advantages of this approach include (1) reduced exposure of nontarget populations to biologicals; (2) vaccine delivery directed specifically to target species; (3) reduced environmental exposure to virus; and (4) no bait delivery system required. Disadvantages include (1) not cost effective; (2) labor intensive and time consuming; (3) need for annual or biannual repetition; (4) sufficient levels of immunity for maintenance of group immunity cannot be achieved; and (5) absence of a rabies vaccine currently approved for use in wild animals in this country.

c. Oral Modified Live Rabies Vaccine

The concept of control of rabies by oral immunization of populations of wild animals is not new (10). The discovery that orally administered attenuated rabies virus (ERA strain) invaded primarily through the lingual and buccal mucosal membranes of mice indicated that oral immunization was possible.The focus of oral immunization trials then shifted to the red fox (*Vulpes vulpes*), due to its prominence as a rabies vector in parts of North America and Europe. Researchers found that foxes developed VNA to rabies after oral immunization with ERA virus (9,15,31,67,145). After determining the efficacy of ERA virus by the oral route, attempts were made to develop an oral bait to deliver the vaccine efficiently under field conditions. In the laboratory, investigators successfully immunized foxes using vaccine-filled sausages, dog biscuits, and fish-flavored plastic bags as baits (6,15,16).

The attenuated SAD and ERA vaccines have performed well in laboratory trials for oral immunization of foxes against rabies, have been applied extensively in the field throughout much of western Europe and Canada,

extensively in the field throughout much of western Europe and Canada, and have achieved significant control and even local eradication of rabies in some endemic areas (114,115,116,121,137,147). Advantages of these conventional modified-live vaccines are (1) genetic stability and extensive field testing in Europe; (2) lack of any genetically-altered virus; and (3) extensive laboratory and domestic animal experience with these vaccines. The disadvantages include (1) unlicensed for use in wildlife in the United States; (2) not orally efficacious in raccoon (*Procyon lotor*) or striped skunk (*Mephitis mephitis*), the primary wildlife reservoirs in the United States; (3) in rare situations, known to cause rabies in nontarget species (e.g., rodents in captive trials); and (4) potential public health risk.

Although live oral rabies vaccine has been used for fox rabies control in Europe and Canada for over a decade, there is residual risk inherent in the use of all live rabies vaccines. Most notable is the potential to cause disease in a wide range of hosts, as well as the potential for contact with the virus in atypical situations such as by ingestion of multiple doses and through accidental routes of administration. Although stringent laboratory safety testing, extensive safety training of involved personnel, and public information programs have minimized the risk, humans have become infected in the laboratory with attenuated rabies from live vaccines intended for wildlife and have sustained considerable neurological damage. Thus the risk remains of rabies infection from the live SAD and ERA vaccines (30).

d. Oral Live Vector Rabies Vaccine

Oral immunization using conventional modified live virus vaccines have been ineffective for the establishment in the United States of an effective control program for rabies. This is especially true when raccoon and skunk populations are involved (7). Recent advances in the development of viral vector recombinant vaccines have made possible the oral vaccination and protection of wild raccoons against rabies (111,142). Captive raccoons fed a vaccinia-vectored recombinant rabies vaccine (V-RG) in a sponge bait developed rabies VNA (0.6-54.0 I.U.) and resisted challenge with street rabies virus (111). Some trials with the V-RG vaccine have already been completed in Europe, with very encouraging results (see section II.B.2 and ref. 91,97), as has a field trial in Virginia (see section III.B.2 and appendix B). The proposed field trial of the V-RG vaccine in Pennsylvania is intended to further test its safety and efficacy.

C. Background - Vaccine Development

1. Biology of Vaccinia

Vaccinia virus, a member of the pox virus family, has been used as a vaccine for almost 200 years since it was introduced by Edward Jenner for immunoprophylaxis against smallpox. Use of vaccinia virus vaccines has resulted in the worldwide eradication of smallpox, completed in 1982.

The International Committee on Taxonomy of Viruses has proposed a classification scheme for the family Poxviridae based upon the observed host ranges. Those poxviruses whose replication is restricted to vertebrates belong to the subfamily Chordopoxviridae. Within this subfamily is vaccinia virus, a member of the Orthopoxvirus genus, a group of morphologically and antigenically related viruses of warm-blooded vertebrates.

The principal members of the orthopoxvirus genus are vaccinia, variola (smallpox), cowpox, monkeypox, camelpox, raccoon pox (RP), volepox, taterapox (of gerbils), Uasin gishu (isolated from a horse in Kenya), and mousepox (ectromelia) (34). Raccoonpox is the only New World orthopoxvirus found in the wild in the Eastern United States.

At least five suggested historical origins of vaccinia virus have been proposed: from smallpox by arm-to-arm passage; from smallpox by adaptation to animals; from cowpox; from smallpox and cowpox by hybridization; and from horsepox (13). Of these proposed origins, horsepox is the most probable. In the 19th century, smallpox vaccines made from horsepox virus were clinically more suitable than vaccines derived from cowpox, thus horsepox vaccines were retained over many years while cowpox vaccines were rejected. European horsepox is now extinct; therefore, the strains of horsepox virus from which vaccinia virus may have originated are now nonexistent. The genomes of the various vaccinia virus strains are very similar to each other but quite different from those of smallpox and cowpox (36,73). Derivation of vaccinia virus from smallpox or cowpox would have required considerable change in the genomic DNA; such transformation of one orthopoxvirus into another is not likely (35). Vaccinia virus thus appears to be a laboratory virus, nonexistent in nature, for which humans are the principal host (but see section V.E.1.c.).

Vaccinia is a dermatropic virus, which upon inoculation into superficial layers of the skin produces a lesion characterized by cellular hyperplasia, viral proliferation, and infiltration of inflammatory cells. The skin lesion caused by vaccinia virus generally progresses from a papule, through a vesicle and pustule, to a crust. There may be transient viremia, but generalized lesions are extremely rare in immunocompetent hosts. Vaccination induces an adequate humoral and cellular immune response. Vaccinia virus is easily cultured and has been widely studied as a typical poxvirus (29). Vaccinia virus can infect a variety of warm-blooded vertebrate hosts experimentally, but is not perpetuated in nature (12).

When smallpox immunizations were commonly practiced, most vaccinia virus vaccine strains were produced at a concentration (e.g., 10^8 pfu/ml) capable of inducing major reactions in 95% of first-time (primary) vaccinees (90). The recommended vaccination procedure involved the use of a bifurcated needle, dipped into vaccine, that was subsequently inserted a minimum of five times into the skin (intradermally) over the deltoid muscle. Primary vaccinees usually developed a vesicle after 3 to 5 days, which, by 9 days, became a pustular area of induration or congestion surrounding a central ulcer. Primary vaccination usually resulted in swelling and

tenderness at the inoculation site, regional lymphadenopathy, and low-grade fever. Successful healing resulted in a small circular scar. The occasional abnormal reactions ranged from a mild local ulceration to vaccine-induced mortality. Vaccinia is to some extent a human pathogen; this potential appears to be aggravated in hosts with eczema and/or immunodeficiencies, and in the very young. In the smallpox eradication campaign, however, there were cases of vaccine-associated encephalitis in apparently normal persons. Routine use of vaccinia virus immunization against smallpox was discontinued in the United States during 1971 and worldwide in 1982, due to the effective worldwide eradication of smallpox (37).

2. Vaccinia Virus As Vector

Following its primary use in smallpox vaccines, recent technical advances have permitted use of vaccinia virus as a cloning and expression vector (75,94). Recombinant vaccinia viruses bearing foreign-protein coding sequences have been widely used experimentally to immunize animals against various diseases (74,76). Vaccinia virus is particularly suitable for expression of foreign genes because its comparatively large DNA genome (185 kilobase pairs) is capable of accepting extra genes readily (88,117,138). The experimental suitability of this virus is also enhanced by its relative nonpathogenicity to animals and humans (22,57,134,135,136). When live recombinant vaccinia viruses expressing surface antigens from human and animal pathogens (including the rabies virus glycoprotein gene) are tested for immunogenicity in animal hosts, the hosts are protected from virulent challenge with the corresponding pathogenic agent without secondary clinical signs attributable to vaccinia (22,28,77,85,95,96,117,118,124).

a. Replication of Vaccinia

Vaccinia virus replicates in the cytoplasm of infected cells and relies on virion-associated enzymes for production of progeny virus. The DNA sequences of vaccinia viruses are similar to sequences in genomes of other orthopoxviruses. The functional genes are in the highly conserved central part of the double-stranded linear DNA genome; arrangement of sequences in the terminal sections of the genome tend to be characteristic of the different species of orthopoxviruses.

Mapping studies have detected genetic mutations and deletions near both termini of the vaccinia genome (4,65,73,86). Variants of the parent virus occasionally contain inverted terminal repeats that are larger than those in the parent virus, and sometimes involve duplication of additional sequences located at the opposite end of the parental genome. It is not clear whether such structures could be derived by a single recombination event between two unmodified genomes aligned in opposite polarity, or in a more complex manner involving mutant DNAs (or different virus genomes) simultaneously replicating in single cells in an animal (86). In tissue culture under nonselective conditions, there is a high frequency of intramolecular recombination (11,33). The probability of recombination occurring naturally in an animal host, however, is extremely low (51).

b. Selection of Parental Strain of Vaccinia

During the worldwide smallpox eradication campaign, the relative ease of vaccinia virus production on calf skin made local vaccine production feasible, even in underdeveloped countries. As a result, variation in the vaccinia virus developed between the various locality strains (i.e., Copenhagen, Elstree, Tashkent).

The origin of the Copenhagen strain of vaccinia virus is not known. Attempts to trace the origin of this strain for the World Health Organization (WHO) publication, "Smallpox and its Eradication," (37) and through direct contact by Wistar Institute with the Staatens Serum Institute, Department of Toxoplasmosis in Copenhagen, Denmark, have been unsuccessful. Records of the Copenhagen strain of vaccinia virus first appear in 1913 in the Annual Reports of the Serum Institute of Copenhagen, Denmark (Dr. Jepson, personal communication to Wistar Institute), and indicate that it was brought from Germany. Dr. Kragh-Anderson, the Vaccine Institute Director in Copenhagen at that time, tried to demonstrate that the Ecuador strain would be more suitable for a vaccine than the Copenhagen strain. The Copenhagen strain, however, was consistently shown to be less virulent in calves, rabbits, and newborn mice. Analysis in 1913-14 showed that the Copenhagen strain was less virulent than the Munchen (Munich) and Hamburg (West German), Dresden (East German), Tours (French), and Christiania (Norwegian) strains. The Copenhagen strain was, therefore, approved for human vaccine use in Denmark and The Netherlands. The New York Board of Health strain, and the Lister (Elstree) strain, both with comparatively very low virulence, were used for human vaccination in the United States.

The particular Copenhagen strain of vaccinia virus used by Wistar as the vector for the glycoprotein gene of rabies virus in the V-RG recombinant virus vaccine was obtained from Professor Kirn of the Virology Laboratory of the University of Strasbourg, France. He obtained it in 1962 from Dr. Triau of Institute Merieux, Lyon, France; who obtained it from Dr. Frankel of the Central Veterinary Institute, The Netherlands; who obtained it from the State Laboratory of Public Health, The Netherlands; which earlier had received it from the Staatens Serum Institute of Copenhagen, Denmark. The Triau material was designated NF 121 (0.5 ml per ampule of Frankel preparation diluted to10^{-1} and titered 7.5 x 10^{8} pfu/ml in embryonated chicken eggs) and was known to produce a cytopathic effect on sheep kidney cells and mouse L cells. Kirn subsequently described a method of plaquing this virus on epidermoid carcinoma (KB) cells (60). The strain was used briefly as a vaccine in Denmark and The Netherlands, and was reported to Wistar to have caused two cases of encephalitis (neither was fatal) in approximately 25,000 vaccinations (unpublished data, Frankel letter to Triau, 1962).

c. Construction of the Live Vaccinia Virus Vector Rabies Vaccine

Utilizing a vaccinia virus as the expression vector, V-RG was constructed by the transfection of vaccinia (Copenhagen strain) virus-infected cells with a vaccinia transfer plasmid containing a rabies glycoprotein cDNA inserted within a cloned vaccinia TK gene (59,143). Insertion of the rabies glycoprotein gene functionally inactivates the vaccinia TK gene. The glycoprotein is noninfectious and nonreactive except as an immunogen for induction of rabies VNA. Inactivating the TK gene has been shown to significantly reduce (attenuate) the pathogenicity of the WR (mouse-adapted) strain of vaccinia (22).

The rabies glycoprotein gene, which has been inserted into the vaccinia virus vector, codes for a single rabies virus-specific protein. The V-RG recombinant virus is genetically stable, retaining the rabies glycoprotein gene during replication in cell culture and serial i.c. mouse passage. If the glycoprotein gene was somehow excised from the vector, it is possible that some portion of the TK gene would be lost in the process and the recovered virus would remain nonfunctional because of the incomplete TK gene. Since the construct is stable, loss of the rabies glycoprotein gene is highly unlikely. A description of the construction of the V-RG virus is contained in appendix A.

d. Comparison of Vaccine With Parental Strain of Vaccinia

The V-RG recombinant virus shares many properties of the parental vaccinia virus. Both parental and recombinant viruses replicate normally in BHK-21 and Vero cells, two cell types which have been employed for large-scale production of conventional vaccines. Both viruses form similar small-size round plaques in cell culture. Comet-shaped plaques characteristic of virulent vaccinia virus strains are not formed by either virus. Flourescent-antibody staining of BHK-21 cell monolayers infected with the V-RG virus reveals that the glycoprotein expressed from the rabies glycoprotein gene sequence is present in the cytoplasm of acetone-fixed cells and at the cell surface. This finding suggests that the recombinant replicates in the cytoplasm of infected cells as does the parental virus (11,86).

The most important characteristic of the V-RG virus that differentiates it from the parental virus is attenuation. Insertional inactivation of the vaccinia TK gene with Wyeth strain decreases virulence for test animals (mice) by i.c. and intraperitoneal (i.p.) routes (22). This decrease in virulence can be seen by comparing diameters of test lesions at sites of inoculation, and the dose of virus required to produce the lesions (21). The diameter of the lesions is smaller and the dose required to create the lesion is larger with the V-RG recombinant virus.

Several experiments conducted by Wistar in immunodeficient mice examined safety and virulence of V-RG virus compared with the parental Copenhagen virus strain. One study compared their virulence when inoculated into immunodepressed 6-week-old female Swiss nude/nude mice via the footpad and i.p. routes. The Copenhagen strain caused lesions at the

higher of the two dose levels (10^8, but not 10^7 pfu) on i.p. administration. These lesions were located in tail epithelium, suggesting that a systemic viremia had occurred with secondary infection of tail epithelium. V-RG virus was not isolated from the blood or liver of test animals, and no tail lesions occurred. No lesions were detected on footpad administration with either virus. Although this data indicated that V-RG may be at least 10-fold less virulent than the parental Copenhagen strain, the data does not provide sufficient information for determinating the extent of the difference. A more recent study further evaluated the characteristics of the V-RG virus compared with the parental Copenhagen derived virus. Severe combined immuno-deficient (SCID) mice and white Swiss mice (ICR) were injected intracerebrally with two dilutions of V-RG and one dilution of parent strain vaccinia virus. The ICR mice were more resistant than SCID mice to both viruses. SCID mice were highly susceptible to the parent virus, but had a 75% survivorship when the V-RG was administered at similar titers. These data indicate both that the V-RG virus is more attenuated than the parent Copenhagen strain, and that most immunodepressed animals survive infection with the V-RG vaccine virus (see appendix E for further details).

Attention to the parental vaccinia virus origin and passage history is important because it was widely believed that the several different vaccinia virus vaccine strains used in different countries during the smallpox eradication campaign contributed to the variation in human complication rates observed (37) (see section V.C.2.). It is clear that there was a difference in the incidence of complications to smallpox vaccination, especially post-vaccinal encephalitis (125). One study in The Netherlands reported one case of post-vaccinal encephalitis in 4,000 primary vaccinates. In contrast, in the United States the incidence of this complication was estimated to be 2-6 per 1,000,000 primary vaccinates (66). The difference in the observed rates was attributed to the strain of vaccinia used, which in The Netherlands was the Copenhagen strain.

The pathogenicity of the Copenhagen strain has been compared to the Bern strain (Germany), the Ecuador strain (South America), and the Elstree (Lister Institute) strain (Great Britain) (101). These studies calculated an index of pathogenicity (number of febrile (38.7°C) days in 100 vaccinations) and demonstrated that the Copenhagen and Bern strains were more pathogenic than the other two. As a result of this and other evaluations, use of the more pathogenic strains declined, so that by 1972 most of the vaccines used in the WHO eradication program were derived either from the Elstree (Lister Institute) strain or the New York Board of Health strain (146).

Post-vaccinal encephalitis was much more common in European countries than in the United States, and more common in the countries of continental Europe than in the United Kingdom. However, there were also differences in the age incidence. While post-vaccinal encephalitis was mainly a risk of primary vaccination in adolescents or young adults in the United States, a higher incidence of encephalopathy in infants under the age of 1 year led to

a recommendation that compulsory vaccination should be postponed until the age of 2 (144).

Although there is no single laboratory test that can satisfactorily evaluate the virulence of vaccinia virus strains for humans, or assess the likelihood that vaccination will cause post-vaccinal encephalitis, investigations (79) in mice and rabbits resulted in the following classification of several strains in terms of their pathogenicity: mildly pathogenic (New York Board of Health and EM-63), moderately pathogenic (Lister, Bern, and Pathadangins), and highly pathogenic (Copenhagen, Tashent and Ikeda) (see section III.B.1.c and appendix D for a discussion of strain differences in nonhuman primates).

To evaluate the rate of serious complications, studies involving more than a million primary vaccinates would be necessary to determine the incidence of post-vaccinal encephalitis, vaccinia necrosum, or eczema vaccinatum. There is also some question as to whether new milder strains would be protective against smallpox (89). Although the exact etiology for many of the reactions (e.g., human post-vaccinal encephalitis) has not been determined, robust experimental animal models were never developed to adequately mimic postulated cause-effect relationships of so-called more virulent vaccinia virus strains in animals.

Post-vaccinal complication frequencies in different countries, and even in the same country, varied considerably from year to year. For example, between 1956 and 1970, Denmark (133) reported 20 cases of post-vaccinal encephalitis per million primary vaccinees using the Copenhagen strain, compared to other strains associated with 9 cases per million vaccinated in the United Kingdom between 1961 and 1970, and to 3 per million in the United States for 1968. No significantly higher rates of mortality from post-vaccinal encephalitis using the Copenhagen strain were reported - 3.3 per million for Denmark, compared to 2.5 per million for Sweden; and 2.2 per million for Great Britain and Wales, where the Copenhagen strain was not used. Moreover, whereas almost a two-fold decrease in post-vaccinal encephalitis was reported for The Netherlands after the 1962 switch from using the Copenhagen strain (used during 1959-62) to the Elstree strain (used from 1963-66), a further five-fold reduction was recorded for the period 1967-70, when only the Elstree strain was used (100). Thus, other factors, such as adherence to accepted contraindications to vaccinia vaccination (e.g., age, eczema, etc.), inadvertent inclusion of allergic proteins, and bacterial contamination in the vaccine, may have played a role in the variation in complication rates. Finally, a direct comparison of different vaccinia virus strains from retrospective epidemiological data, at least as regards potential human health effects, may be almost impossible to obtain due to (1) the relatively low rate of serious post-vaccinal complications overall, (2) the lack of consistency in case definitions required for diagnostic reporting following vaccinia immunization, and (3) the frequent omission of denominators for number of primary vaccinees and the population ages compared.

3. Comparison With Other Rabies Vaccines

Classical modified-live rabies vaccines occasionally cause actual rabies in a vaccinated animal. There is no possibility, however, of vaccine-induced rabies occurring in animals exposed to the V-RG virus. This vaccine contains only the rabies glycoprotein gene, and no part of the infective virion. The V-RG virus induces high levels of circulating rabies VNA in raccoons after non-oral inoculation (142), and when administered orally in baits (111). Protection for up to 18 months is also possible with V-RG against antigenically diverse street rabies virus as well as against such related lyssaviruses as Duvenhage virus (18,143) (table 1).

4. Delivery Systems

For an oral wildlife vaccine to be effective, it must first be discovered and consumed by a large proportion of the target species population. This usually requires that some type of attractive bait be associated with the vaccine. Characteristics of an ideal bait include (1) immediate consumption by an animal upon discovery, rather than stored for future use; (2) unattractiveness, or repellency, to nontarget species, including humans; (3) the potential to add a biomarker; (4) noninterference with the efficacy of the vaccine; (5) low in cost and easily available; and (6) produces no adverse effects in consumers or the environment.

Bait delivery systems previously used have contained the following components: a sterile vaccine compartment, either synthetic sponge, cube foil sachette, or paraffin ampule; a biomarker, such as tetracycline, to verify consumption and uptake; a scent/flavor attractant of either banana, shellfish, grape jelly, turkey gravy, or feta cheese; and a bulk carrier, such as polyurethane, tallow, and paraffin; waste chocolate; or fish meal and polymer (16,55,56,110, 114,116). Most of these systems derive from West German and Canadian prototypes developed for foxes. Several recent U.S. field studies have involved the use of placebo baits (without vaccine) to estimate target species contact. For example, 500 placebo baits of several attractant types were dropped three times in 1 year by light aircraft at a density of 1.2 baits/ha at five areas (each approximately 4 km^2) in a raccoon-rabies epizootic zone of central Pennsylvania. Raccoons were then live-trapped, or carcasses were obtained from trappers and hunters, and examined for biomarker uptake. Results from this trial demonstrated bait acceptance rates from 26 to 76% (110). In a Maryland study using mackerel-flavored sponge baits loaded with iophenoxic acid and tetracycline, biomarkers were detected in 63% of the raccoons subsequently captured (99). Studies in South Carolina and Virginia using fishmeal polymer placebo baits with biomarkers demonstrated at least 78% disturbance rates after 48 hours and raccoon biomarker uptake in the 40 to 100% range (45,47).

These various placebo bait trials obtained data on bait contact rates as a measure of the adequacy of the delivery system. Because these trials necessitated the subsequent capture and examination of animals, they also provided (1) a resource inventory on site fauna; (2) information on raccoon population dynamics, movements, and habitat utilization; and (3) baseline

seroprevalence of rabies, raccoonpox (RP), and other selected diseases prior to proposed initiation of vaccine-bait placement.

Outside the United States, several programs attempting to control rabies in wildlife provided direct or indirect information on the efficiency of bait delivery systems (148). In Ontario, Canada, aerial distribution of 80,000 baits containing ERA strain rabies virus and tetracycline produced encouraging results in several wildlife target species (56). A sponge bait was used, flavored with liver slurry, fish oil, or fruits and wrapped in a polyethylene bag along with tetracycline-laden meat. Results of the study indicate that 74% of the foxes, 54% of the striped skunks, 43% of the raccoons, and 80% of the coyotes that were captured contained biomarker at necropsy (5).

In several European countries, distribution of oral rabies vaccine in baits attempted to control rabies epizootics in foxes. Beginning in 1978, workers in Switzerland distributed chicken head baits containing the SAD strain rabies virus vaccine and a tetracycline biomarker over a 5,000 km^2 area. This large-scale effort resulted in the immunization of 60% of the fox population against rabies and prevented the spread of a rabies epizootic into the study area. Between 45 and 80% of the foxes were estimated to be positive for the bio-marker, the specific value dependant upon bait accessibility and density (121).

Field trials conducted in the Federal Republic of Germany used chicken heads or fat and fishmeal baits and a different rabies virus vaccine (SAD/B19). Between 1983 and 1986, the distribution of 2.4 million baits containing the attenuated SAD/B19 virus resulted in the immunization of more than 70% of the fox population in the areas baited. Bait contact rates, based on tetracycline positive bone samples, ranged from 57 to 90%, and the correlation between the presence of rabies antibody and tetracycline positive status ranged from 43 to 79% (115). Similar field trials began on a smaller scale in Italy in 1985, and in Austria, Luxembourg, Belgium, and France in 1986. By the end of 1988, more than 6 million live SAD/B19 vaccine-laden baits had been distributed in Europe, with local "eradication" of rabies from baited areas where rabies had been endemic for the last 40 years (116) (see section III.B.2).

D. Relevant Federal Regulations

The Virus-Serum-Toxin Act (VSTA) (21 U.S.C. 151 *et seq.*) prohibits the preparation and sale or importation of virus, serum, toxin, or analogous products intended for animals (domesticated or wild) that are worthless, contaminated, dangerous, or harmful. The federal regulations implementing VSTA (9 CFR 103.3) require authorization by APHIS before an experimental biological product can be shipped for the purpose of treating limited numbers of animals as part of an evaluation process. APHIS must first determine that the conditions under which the experiment is to be conducted are adequate to prevent the spread of disease. The procedures set forth in the request for authorization to ship the experimental product must also be approved by

APHIS. Such procedures may involve special restrictions or tests when they are deemed necessary or advisable. Applicants may also be required to provide additional information in order that the Agency can assess the product's impact on the environment. Under the provisions of 9 CFR 103.3, permission from appropriate state animal health officials is required before APHIS may approve field trials. Wistar has obtained permission from the appropriate agencies of the Commonwealth of Pennsylvania for conducting the proposed field trial.

Because a vaccine field test involves the release of an experimental biological product into the environment, and because that release is permitted by a federal agency, the procedural requirements of the National Environmental Policy Act (NEPA) (42 U.S.C. 4321 *et seq.*) apply. Regulations for implementing NEPA have been established by the Council on Environmental Quality (CEQ) (40 CFR 1500-1509), USDA (7 CFR 1b), and APHIS (44 FR 50381-50384 and 44 FR 51272-51274). These regulations require that an environmental analysis be conducted when a major federal action is proposed. This analysis may involve a detailed environmental assessment of the federal action to determine if there is any significant impact on the environment.

Procedures that insure the safety of laboratory personnel handling biologicshave been developed and implemented under the U.S. Public Health Service guidelines, "Biosafety in Microbiological and Biomedical Laboratories" (HHS, Publ. No. (NIH) 88-8395). The handling of experimental animals follows the USDA guidelines on "Policy on Humane Care and Use of Laboratory Animals" (9 CFR Chap. 1, SubChap. A, Parts 1-4).

IV. Purpose and Need for Proposed Action

A. Significance

In the developing world, cases of human rabies continue to be a major concern of public health authorities and the general population. For example, during 1987 in India there were an estimated 12,000 to 20,000 human deaths from rabies and 250,000 post-exposure treatments (123). This general situation exists even though the Wistar HDCV live attenuated vaccine for humans has proven very effective in preventing the disease in developing countries when used either as a prophylactic or post-exposure treatment.

Transmission of rabies to humans is usually by the bite of a domestic or wild animal. In the United States, effective vaccination programs in domestic dog and cat populations have reduced the incidence of rabies in those animals by 85% over the last 30 years (8). The incidence of rabies in wildlife populations during the same period, however, has more than tripled.

Public health, veterinary, and wildlife officials have in recent years been concerned by the expanding epizootic of raccoon rabies in the mid-Atlantic and Southeastern United States. Enzootic raccoon rabies, first recognized in Florida during the 1940s, has increased in geographic distribution at the rate of approximately 25 mi per year; by 1976 it included much of Georgia and parts of Alabama and South Carolina (54). Raccoon rabies was first reported in West Virginia in 1977, and along the Virginia-West Virginia border in 1977, both areas several hundred miles north of the endemic disease front. By 1983 the mid-Atlantic states (West Virginia, Virginia, Maryland, Delaware, Pennsylvania, and the District of Columbia) were reporting a substantial annual increase of cases of rabies in raccoons (24,98). During 1988, Pennsylvania led the nation in reported animal rabies cases, most of which were in raccoons (45).

Increasing prevalence of raccoon rabies is of particular concern in the mid-Atlantic states, due to both the relatively high population density of raccoons and their close proximity to urban-suburban human and domestic animal populations. Reported cases of rabies from domestic animals and humans are expected to increase as raccoon rabies becomes more prevalent. The development of an effective wildlife vaccine could have a major impact on efforts to control the spread of rabies.

The economic cost of rabies in the United States can only be estimated, with post-exposure treatment of humans representing a small part of the equation. Approximately 20,000 people are treated on a yearly basis at a cost of $600 to $800 per person, and this value only includes the cost of the human diploid cell rabies vaccine and the human rabies immunoglobulin (30).

Further costs of rabies may be less tangible but still substantial. These include (1) recreational value and revenue losses associated with the

restriction of activities at parks and wildlife areas due to concerns about safety or liability, (2) loss of revenue from decreased issuance of hunting licenses and the impact on the associated sportsman industry, (3) potential impacts on threatened or endangered species in a rabies epizootic or enzootic area, and (4) costs of follow-up epidemiological investigations of each human exposure. One estimate for the State of Georgia indicated that the total cost of controlling and treating rabies for 1 year was $7.4 million, or a per capita cost of $1.24. Extrapolating this value to the entire United States suggests that rabies may cost the country in excess of $300 million per year (40).

B. Background Of Proposed Action

Several vaccines have been employed in the past for control of rabies in wildlife populations. Non-oral routes of delivery of these vaccines to wildlife, however, have proven not to be cost effective and as such are infrequently used at present. Research on vaccine delivery systems for wildlife since the late 1970s has focused on oral methods associated with baits (148). Oral rabies vaccines containing live attenuated virus have been effective in immunization of foxes, but have been ineffective by this route in raccoons or skunks (7). Considerable concern about the possibility of vaccine-induced diseases when attenuated rabies viruses are used in wildlife vaccination programs has led to a search for recombinant vaccines that cannot produce rabies infections (30,111). The V-RG vaccine developed by Wistar contains an antrax virus with a gene that encodes for a rabies surface protein inserted into the orthopox genome. Considerable laboratory and field evaluation of the V-RG vaccine has preceded the request by Wistar for a field release in Pennsylvania.

1. Laboratory Safety Trials

a. Target Animal

The V-RG vaccine has been inoculated into, or consumed in bait by, a large number of raccoons in a variety of laboratory experiments conducted by Wistar. Information on the safety of the vaccine for raccoons can be obtained from most of these studies. When adult raccoons were administered 1 ml (10^8 pfu) of V-RG recombinant virus either by consuming a vaccine-laden bait or by oral infusion, they invariably developed rabies VNA and suffered no ill effects. They also usually survived a challenge with rabies virus (111). V-RG virus has been recovered at low titer (10^2 pfu/ml) only from buccal mucosa, tonsils, and submandibular/parotid lymph nodes of orally immunized raccoons and only during the first 48 hours after vaccination. No viremia was detected during the 14 days post-inoculation, and no gross or histopathologic lesions attributable to bacterial or viral infection were found in any of the sampled tissues (109). There were no significant differences in cerebrospinal fluid (CSF) cytology between nonimmunized and V-RG vaccine-recipient raccoons, no evidence of CNS invasion by V-RG recombinant virus, and rabies VNA was not detected in the CSF of any animal (48).

Contact transfer of V-RG virus between male-female pair combinations in cage trials has been demonstrated when one of the pair is orally immunized and the other is nonimmmunized. The nonimmunized animals developed low levels of rabies VNA and survived rabies virus challenge, as did all of the orally immunized raccoons (table 2). In another experiment, three suckling raccoons approximately 3 to 4 weeks old were placed with their mother immediately after the mother received 1 ml (10^7 pfu) of V-RG recombinant virus by mouth; all animals remained healthy, seroconverted within 28 days, and survived peripheral rabies virus challenge. Thus, in certain special cases raccoons that do not consume V-RG vaccine in baits may still come in contact and be infected with the virus, though such exposure does not lead to adverse effects.

Pregnant raccoons have been immunized with 1 ml (10^7 pfu) of V-RG recombinant virus within 30 days prior to parturition and have given birth to healthy litters of normal size. All littermates had levels of rabies VNA comparable to that of the adult females, suggesting the occurrence either of passive transfer of maternal antibody or active infection *in utero,* though no virus could be isolated from the young animals (109). Other pregnant raccoons have been given V-RG vaccine at various times prior to parturition, with no subsequent abortions. Development of VNA in these offspring was maternally derived, as only the mothers demonstrated a subsequent anamnestic response to inactivated vaccine (107). These data indicate that even fetal raccoons suffer no ill effects from the infection of their mothers with the V-RG virus.

b. Nontarget Animals

When the V-RG vaccine is placed in baits and offered to wildlife under natural conditions, contact by nontarget animals cannot be prevented. Nontarget mammalian wildlife species potentially at risk for vaccine contact were identified by placebo trials at the proposed Pennsylvania field trial site and previously at other locations in the United States, and during vaccine field trials in Europe. Various domestic animals, commonly hunted mammals, and some avian predators and scavengers, were also considered to be at risk. Over a period of 10 years, most of those species in North America and Europe identified or postulated to be at risk of infection with V-RG virus have been maintained in the laboratory and exposed by oral and other routes to V-RG virus. Appendix C lists 44 species of birds and mammals tested to date for the effects of V-RG exposure. Individuals of these species represented both sexes and a wide variety of ages and body conditions, and were determined to be sero-negative before exposure. Of the 320 individual animals inoculated with V-RG vaccine, none have demonstrated pox-like lesions, morbidity, or mortality that could be attributed to infection with the V-RG virus. Contact transmission of V-RG virus between vaccinated and nonvaccinated animals housed together almost always does not occur. The very rare exceptions to this nontransmission have occurred in unusual conditions that also are rare (109). When virus is present, it is found only for the first several days after exposure in oral areas such as

tonsils, buccal mucosa, and retropharyngeal lymph nodes (128). These laboratory trials demonstrate the innocuity of V-RG in all tested nontarget species (see appendix C for further details).

c. Primates

Although experiments that directly test the infectivity and subsequent effects of the V-RG vaccine for humans cannot be conducted, there are several indirect sources of information concerning the safety of this vaccine for exposed humans. One source of information could be the safety record of laboratories that have worked with the V-RG virus. Ordinarily, lab personnel working with infectious materials or animals are protected by immunization and by procedures and equipment that minimize risk. Thus, it is not surprising that V-RG virus has been completely safe for humans in laboratory situations. Potential nonlaboratory exposure of humans in the various European field trials of V-RG vaccine has been considerable, with no program in place that monitors antibody levels of residents before and after the field trials. There have not been any reports, however, of increased incidence of sickness in the field trial areas that could be attributable to the V-RG vaccine.

Another source of information involves the use of nonhuman primates as models for vaccine safety and efficacy in humans. Studies were conducted by Wistar employing squirrel monkeys and chimpanzees as surrogate human hosts for theV-RG virus. The first study involved dermal inoculation of 26 adult squirrel monkeys (*Saimiri sciureus*) and was designed to assess the possibility of contact transmission of the V-RG virus to unvaccinated controls and to compare V-RG with two strains of vaccinia virus — the parental Copenhagen derivative and the New York Board of Health (NYBH) strain. All vaccines were diluted to contain $10^{8.0}$ pfu/ml. The animals were assigned to one of three groups, with animals in each group inoculated at three sites by skin scarification. Two controls were inoculated with phosphate-buffered saline only. On day 7 post- inoculation and at weekly intervals thereafter, samples of blood and oral and fecal swabs were collected. The blood was tested for virus neutralizing antibody, and the oral and fecal swabs were subjected to virus isolation procedures. Two vaccinates from each group were euthanized and necropsied at days 7, 14, 21 and 60 post-vaccination. One control was euthanized at day 7 and the other at day 60 when the study was terminated. During the 60-day period post-vaccination, no animal exhibited evidence of abnormal physical or behavioral traits. All vaccination sites, except in the saline controls, developed slight erythema, which progressed to moderate local inflammation by day 5 post-vaccination. Lesions of the NYBH strain and the parental Copenhagen virus became erosive, while lesions of the V-RG virus were confined to the epidermis. No abnormal changes were noted in any organ at necropsy. Rabies VNA was first detected 7 to 14 days after inoculation only in animals directly receiving the V-RG virus.

The second study involved eight chimpanzees (*Pan troglodytes*) that were orally administered 1 ml ($10^{7.2}$ pfu/ml) of V-RG. Three sentinel non-immunized animals were also included in the study. All animals were caged separately in the same room. Blood samples were collected on days 0, 14, and 30 for VNA analysis. Fecal samples were collected from each animal, daily for the first week, twice in week 2, and once during weeks 3 and 4, for virus isolation. Oral swabs were collected on days 14 and 30. Approximately 66 days after the first vaccination, three sero-negative animals were added to the experiment and all animals except the original sentinel controls were given $10^{9.0}$ pfu(1 ml) of V-RG orally. This second stage of the study was initiated because of the inadequate immunological response to the first vaccination. No virus was recovered except at day 6 from one chimpanzee that had a preexisting bacterial dermatitis. This animal developed a low-grade fever, depressed appetite, and swollen lymph nodes, whereupon immediate treatment with antibiotics quickly eliminated evidence of the infection. Of the other 10 vaccinates, after the second vaccination 6 developed high VNA titers to rabies and 2 had relatively low titers. Particular attention in this second phase of the experiment was given to the potential development of oral lesions. Oral and fecal swabs were taken as before, and blood samples were collected on days 14 and 30. Blood samples were also obtained from humans working in direct contact with the vaccinated chimpanzees. No vaccine virus was recovered from any of the oral or fecal swabs in the second phase of the trial, and sentinel control animals and human animal caretakers remained negative for V-RG virus antibodies (see appendix D for further details).

These experiments demonstrate, by deliberate transdermal inoculation of theV-RG virus into primates, that indirect human exposure to V-RG that might occur via bite or body fluids from a recently vaccinated animal is unlikely to produce adverse clinical signs.

2. Field Trials

Field trials represent the logical extension of laboratory safety and efficacy evaluations to more complex natural ecosystems. Laboratory experiments have clearly demonstrated the innocuity of the V-RG virus for nontarget animals. Environmental conditions in the field, however, are much more variable and unpredictable than conditions in the laboratory. Thus the safety of the V-RG for nontarget animals must be demonstrated under field conditions before the vaccine can be licensed for general use. The following field trials were designed to document: the incidence of vaccine virus in nontarget animals and its consequences, contact rates with vaccine and bait, and/or the likelihood of accidental transmission of vaccine virus to humans.

a. Argentina

Although technically not a field trial, the following experiment has been reported in the popular press as such (51), and thus will be considered in this section.

In 1986 a containment trial of the V-RG vaccine in cattle was initiated in Argentina at the Pan American Health Organization's (PAHO) Centro Panamericano de Zoonosis facilities, with Wistar as one of the collaborators. One of the objectives of this trial was to document the degree of contact transmission of the vaccine virus from inoculated to noninoculated animals.

Ten cows were inoculated subcutaneously in the neck with 1 ml of V-RG (10^8 pfu per animal) and 10 cows were vaccinated with the same product by i.d. scarification in a previously shaved area on the neck. Each group of 10 cattle was kept in close contact with 10 nonvaccinated cows, with all animals confined in barns. In 1986 this trial was terminated by the Government of Argentina, and the sera collected from cattle and exposed humans were tested for rabies antibodies. The initial report released by the government claimed that there had been transmission of the V-RG vaccine from vaccinates to contact controls and to humans working with the cattle. These results were disputed by Wistar and PAHO, and the Argentine government appointed a commission to oversee retesting of the sera. The commission included representatives of the Argentine government, PAHO, and an independent representative from the World Health Organization. The retest confirmed the PAHO position that vaccinated cattle all developed antibodies to the rabies glycoprotein, that no seroconversion occurred in response to rabies antigens in 20 unvaccinated cattle held as contact controls, and that the human contacts also failed to develop rabies antibodies. The conclusion was that there was no spread of the V-RG vaccine from vaccinated cattle (63).

b. Belgium and France

In Europe, rabies is a major public health concern that involves both human and wildlife populations. The principal wild animal vector and reservoir in this region is the fox. Programs to control rabies spread from foxes have previously included inactivated rabies vaccines, but their effectiveness has been unacceptable when orally administered. A new recombinant-derived vaccinia virus vaccine expressing the rabies glycoprotein (V-RG) was evaluated in European field trials prior to proposed trials in the United States.

Before the European field trials were initiated, the safety of the recombinant rabies vaccine was tested in standard laboratory animals, domestic animals, and in wild mammals (including fox) and birds (19). Routes of administration included intradermal, subcutaneous, intramuscular, and oral. The results of these laboratory tests indicated that the live vaccinia vector vaccine virus was not excreted from test animals and that horizontal transmission from vaccinated animals to contact controls did not occur, except in a few cases where vaccinated animals had bitten controls very soon after vaccination.

Efficacy of the experimental rabies vaccine was evaluated by both serological tests and by challenging vaccinates with virulent rabies strains.

Effective immunization following oral administration of the recombinant vaccine was demonstrated in foxes, raccoons, skunks, dogs, and cats (17,18,20,111,129, 130). Protection of foxes and their cubs was shown to be dose-dependent following vaccination by the oral route. The 100%-protective dose (PD100%) was thus estimated to be 10^7 pfu, and immunity was found to persist for at least 18 months in adult foxes. Once these preliminary safety and efficacy studies were successfully completed, field trials were initiated to examine both the safety and the potential of the V-RG vaccine for rabies control in nature.

First Trial — Belgium. The first limited field trial of the vaccine was authorized by the Belgium Ministry of Public Health on September 23, 1987. The trial site was a 27 km^2 military field isolated from domestic animals and the public, and located in an area of the Ardennes province (Marche-en-Famenne) where fox rabies is commonly found. On October 17 and 18, 1987, 250 baits (chicken heads) with implanted capsules containing liquid vaccine and a tetracycline biomarker were manually placed over a 6 km^2 area situated in the center of the military field and surrounded by a nonvaccinated buffer zone.

During the subsequent 3-month observation and trapping period, no abnormal morbidity or mortality was noted among animals, either in the vaccinated area or in the nonvaccinated buffer zone. There were 145 small mammals trapped in the baited area; 4 had eaten baits (based on presence of the biomarker), and none of the 4 animals showed any lesion that could be related to poxvirus infection. Antibodies against poxvirus were not detected in the sera from13 badgers, 18 boars, 20 deer, 8 porcupines, and 16 foxes captured in the study area. Antibodies against rabies were also not detected in three boars killed at the vaccination site (97).

Second Trial — Belgium. The Belgium Ministry of Public Health on September 21, 1988, authorized a second and larger field trial, which was also located in the Ardennes region. Between October 24 and November 5, 1988, approximately 6,000 vaccine-bait packages were manually distributed throughout a 450 km^2 field. A 20 km^2 area within the larger site was chosen for extensive survey. The vaccine-bait package consisted of a vaccine container inside a bait unit inside a plastic bag. The 2.5 ml of liquid vaccine contained 10^8 $TCID_{50}$ of V-RG. The 5x3x2 cm bait unit was a polymer containing fish meat, fish oil, and 150 mg of the biomarker tetracycline.

In this trial, bait disturbance rates were approximately 65% by day 15 and exceeded 94% by day 30. Between November 10, 1988, and March 30 1989, 54% of the foxes captured in the area had eaten one or more of the baits as determined by tetracycline uptake. Three other animal species — wild boar, stone marten, and stray cat — also demonstrated significant biomarker uptake. There was no abnormal morbidity or mortality reported among domestic or wild animals during a 7-month period following distribution of baits. Of 226 animals (fox, stone marten, marten, ermine, polecat, stray cat, wildcat, hare, squirrel, hedgehog, rodent, deer, mouflon, and boar) hunted,

captured, or found dead within the baited area, none showed any lesions that could be related to poxvirus (32).

Third Trial — Belgium. This large-scale trial involved the dropping of bait from helicopters onto 25 contiguous districts (including the 4 districts vaccinated in 1989) during three time periods. In October of 1989, 24,960 baits were dropped over 2,200 km^2; in March-April, 1990, 30,000 baits were dropped; and in October-November, 1990, 175,000 additional baits were dropped into the test site. The vaccine-bait package was the same as that used in the previous trial. Preliminary data from this trial indicate a 62% biomarker uptake (47/76) in adult and juvenile foxes. It is estimated that 80% of the vaccine-bait packages were either consumed or removed by animals in the first 30 days. The incidence of rabies in foxes in the treated area has significantly decreased over the course of the trial and the population density has remained relatively high (148).

First Trial — France. On January 20, 1988, the French Commission for Biomolecular Engineering of the Ministry of Agriculture gave authorization for a limited field trial on a 53 ha military field at Mars le Tour (Departments of Meurthe and Moselle). In November 1988, 174 specially developed baits, similar to those used in Belgium, were manually distributed within a 17 km^2 area, and 187 control (nonvaccine) baits were scattered in a control area of the same size. The vaccine and control areas were separated by a 6 km^2 buffer zone. Within 15 days of bait placement, 90% of the baits had disappeared. Wildlife censuses before and after vaccine placement showed no significant population changes in either the vaccinated or control areas. No increase in morbidity or mortality was noted among the entire fauna. Examination of 77 carnivores, rodents, and birds trapped during December 1988 did not reveal any lesions that could be related to poxvirus (32).

Second Trial — France. In October and November 1989, additional large-scale field trials were carried out in the Departments of Meuse, Meurthe, and Moselle. About 6,400 baits were dropped from helicopters into open fields where rabies was enzootic. The average site was approximately 750 km^2, with no control zones reported. Preliminary data from this trial indicate an absence of abnormal sickness or death in animal populations in the study area. Examination of 226 animals collected post-treatment indicate an absence of pox-like lesions and an overall biomarker uptake rate of 19%. The biomarker was found, however, in more than 70% of the target fox population sample. Antibodies against rabies have been detected in over 80% of the testedfoxes (32).

Third Trial — France. In March and April, 1990, a very large-scale field trial was initiated in the Departments of Liege, Luxembourg, and Namur. Approximately 600,000 baits were dropped into the area from helicopters. Results of this trial are pending.

Conclusions. These field trials of an orally administered live recombinant vaccine for rabies in Belgium and France can be characterized as demonstrating the absence of adverse reactions in any animal examined to date.

There has been no observed increase in mortality or morbidity among domestic or wild animals, nor have pox-like lesions attributable to V-RG been detected in any animal examined to date. Human populations adjacent to the study areas, as well as personnel involved in the field trials, also have not demonstrated any adverse effects attributable to the V-RG vaccine (32).

c. Virginia

The first field trial in the United States of the V-RG rabies vaccine was initiated in August 1990 on Parramore Island, Virginia. The request to conduct this field trial had been evaluated by USDA-APHIS and an Environmental Assessment (EA) was prepared. A Finding of No Significant Impact (FONSI) was issued on April 14, 1989, indicating that the vaccine field trial would not have a significant impact on the quality of the human environment. Subsequent authorization was obtained from the Commonwealth of Virginia and the owner of the island (The Nature Conservancy), allowing the trial to begin in late summer 1990. As the first United States field trial of a genetically engineered wildlife vaccine, considerable scrutiny was directed toward the potential impact of the vaccine on the ecosystem and its safety for wildlife and humans. A variety of federal, state, and local governmental agencies, as well as numerous environmental and animal rights organizations and private individuals, had input into the design of this field trial.

The field trial was conducted on Parramore Island, a barrier island in the Atlantic Ocean off the coast of the Eastern Shore of Virginia. Located 7 km from the mainland, the island was 12.8 km x 0.2-2 km in size and contained upland forest, scrub thicket, salt marsh, and open dune habitats. Surveys of the island wildlife preparatory to the field trial were begun in October 1987, and indicated that the principal mammals on the island were two carnivores, red fox and raccoon; one ungulate, white-tailed deer; and four small rodents, rice rat, house mouse, meadow vole, and Norway rat. Raccoon density was estimated at one per 2.7 ha, which was quite high compared to published estimates for other sites in the United States (84).

(1) Protocol

On August 20, 1990, 3,120 vaccine-bait units were distributed by hand in six areas within the 312 ha study area. These units were composed of a polyethylene bag containing a polymer cylinder and a slurry. The cylinder was made of fishmeal polymer bait containing a tetracycline biomarker. Inside the cylinder was a paraffin ampule containing 10^8 pfu of V-RG fluid vaccine. The slurry contained water, sucrose, egg yolk, vegetable oil, homogenized blue crab, and the biomarker SDM, and was formulated to increase the attractiveness of the unit for raccoons. Each site containing a vaccine-bait unit was checked daily for 14 days and the condition of the site and the unit recorded. Adjacent areas without vaccine-bait were established as control surveillance areas. Wildlife trapping was conducted daily in both vaccination and surveillance areas for the 14 days of vaccine-bait placement, and then atbi-weekly intervals for the succeeding 12 months. Captured animals

were either sedated for obtaining blood and other samples and then released, or euthanized and samples obtained for histopathologic and biomarker examination.

(2) Field trial results

Approximately 50% of the vaccine-bait units showed evidence of animal contact within 48 hours of placement, and more than 90% were disturbed by the 5th day. Tracks, scat, chew marks, and other animal signs indicated that raccoons were involved in most site and bait disturbances. Over the next 5 months (through January 31, 1991), 234 individual raccoons were captured in both vaccination and surveillance areas, and another 326 captures represented recaptures. None of these animals were remarkable upon physical examination for lesions suggestive of poxvirus. During this period, all raccoons captured from surveillance areas were negative for rabies antibody. Multiple-capture raccoons from the vaccination areas demonstrated a 52% seroconversion rate, indicating apparent contact with the vaccine. A comparison of the mean weight, age, and sex ratios of all raccoons with rabies antibody to those that were antibody negative revealed no statistically significant differences, suggesting that contact with the vaccine and subsequent infection had no detectable effect on these population parameters. In the first 5 months of the field trial, 221 small mammals from both vaccination and surveillance areas were captured and tested for rabies antibodies, with negative results.

The biomarker tetracycline contained in the fishmeal polymer surrounding the vaccine ampule is deposited in the bones and teeth of feeding animals and is detected by post-mortem examination. In the first 30 days of the trial, 92% of the tested raccoons from the vaccination areas were tetracycline positive, whereas none of the raccoons from the surveillance areas (without vaccine-bait) were tetracycline positive. The biomarker SDM that was added to the bait slurry first appears in the blood of animals within 24 hours of consumption and remains detectable in the blood for 6 days. Approximately 78% of the raccoons captured in the vaccination areas and tested in the first 6 days after placement of vaccine-bait were positive for the presence of the biomarker. All raccoons captured in the surveillance areas and tested in the first 6 days were negative for the biomarker. High incidences in raccoons of both biomarkers in the vaccination areas indicates a high contact rate with the vaccine-bait.

Vaccine virus has been isolated only from tonsil tissue of two raccoons on the 2nd and 4th days after vaccine-bait placement. Oral and fecal swabs collected in the first 6 days of the trial from raccoons were all negative for the presence of vaccine virus. These results from virus isolation attempts suggest a low likelihood of transmission by bite of the vaccine virus from infected to noninfected animals.

The 25 researchers associated with the first 2 weeks of the field trial have remained healthy with no suspected vaccine-induced lesions or morbidity. Serum collected from some of these individuals before and after the

vaccine-bait placement period and tested for antibody to the vaccine virus has failed to demonstrate a rise in serum antibody. Some members of the local human population were also bled for serum before and after the baiting period, and tested negative for the presence of antibodies to the vaccine virus.

(3) Supplemental experiments

To provide additional information on the species of animals contacting the vaccine-bait, 100 tracking pits with a vaccine-bait unit in the center were established in the six vaccination areas. These sites were checked daily over a 2-week period for evidence of animal disturbance. Based on the identification of animal tracks, scat, and other sign, raccoons were responsible for 76% of the disturbances and consumption of vaccine-bait.

The rate of vaccine virus deterioration under field conditions was monitored. Fourteen vaccine-bait units were placed in each of several screened enclosures to minimize direct animal molestation. Enclosures were placed at sites in various habitats ranging from full sun to full shade. A vaccine-bait unit was removed daily for 14 days, frozen, transported to the Wistar laboratories, and tested for virus concentration. Weather and invertebrate activity was detectable on the baits, but the interior wax ampules containing the vaccine were not damaged. Maximum loss of virus titer over the 2-week period was minimal, with only a 10-fold drop observed at the full-sunlight sites (from $10^{8.6}TCID_{50}$ to $10^{7.7}TCID_{50}$) and smaller declines at the other sites.

Radio collars with activity and mortality modes were placed on raccoons before and during the first 14 days of the field trial to determine their movements. Radiotelemetry monitoring of these animals was conducted weekly for the first 5 months of the trial and is continuing. At the end of August 1990, 35 functional radio collars were on raccoons. By the end of January 1991, four of these animals had died and nine had lost their collars. Of the remaining 22 raccoons with radio collars, 20 were located in January, indicating minimal long-term movement of these raccoons off the island.

Post-mortem examination of raccoons revealed no gross lesions or other characteristics that could be associated with a poxvirus etiology.

(4) Conclusions

In the first 150 days of this island field trial of the V-RG vaccine, adverse impacts to humans, wildlife, or other aspects of the environment that could be attributable to the vaccine have not been observed. There has been no evidence of V-RG virus transmission from animals consuming the vaccine-bait mixture to other animals, nor is there evidence of increased mortality and morbidity in the target raccoon population (see appendix B for additional details).

3. Preliminary Pennsylvania data

a. Placebo bait trials

Placebo (no vaccine) bait acceptance trials were initiated on the Shingle Mill Road study area of SGL#13 during the autumn of 1989. Ninety experimental fishmeal polymer bait units containing empty wax ampules (no vaccine) were hand-distributed within the study area at a density of 1 to 5 baits per hectare. Bait disturbance and contact was assessed daily along the single 6 km transect line for 3 days.

In the first 24 hours after bait placement, 29% of the baits were missing, and more than 70% were missing by the end of 72 hours. The remaining baits showed no evidence of disturbance by small mammals. The pattern of bait contact and animal sign, as well as consideration of the available mammalian fauna, suggest that small-bodied carnivores such as fox and raccoon were primarily responsible for bait molestation.

b. Parasitological and pathologic survey

Beginning in August, 1989, animals found dead in the field have been examined on site for parasites, histopathology, and general post-mortem parameters. Hunter-killed or live-trapped and euthanized (intravenous sodium pentabarbital) animals that have been obtained from the study area have similarily been examined. Tissues collected include samples of brain, bone, and other major organ systems and were examined in the laboratory for routine pathology. Fifty-nine specimens have been collected to date (February 1991), including 16 porcupines, 12 raccoons, 10 deer mice, and smaller numbers of short-tailed shrews, masked shrews, red-backed voles, chipmunks, red squirrels, grey squirrels, flying squirrels, cotton-tail rabbits, coyotes, and mink. These specimens are being held as reference material for histopathologic comparison with specimens obtained before, during, and after vaccine-bait placement (see section II.A.3).

Examination of the 59 currently available pre-trial specimens revealed unremarkable histopathology and parasite load. One raccoon, however, was determined to be rabid. All specimens were also examined for naturally occurring background levels of the tetracycline biomarker, with negative results.

V. Alternatives

A. No Action

Under the federal no action alternative, a permit would not be issued, thus prohibiting a field testing of the product. The basis for this denial of a permit under 9 CFR 103.3 would be a finding of lack of safety related to the experimental biologic. Such a finding, and the denial of the permit request, would require Wistar to consider two options: (1) conduct further research and development to document or improve the safety of the vaccine, or (2) terminate development of the V-RG vaccine. Both options would significantly delay the eventual control of the current raccoon rabies epizootic in the mid-Atlantic states and lead to additional costs in wildlife mortality and human endangerment.

B. Approval Of Field Test

The federal action in this proposal would be the authorization of the shipment of an experimental biological product, V-RG recombinant virus vaccine, for the purpose of evaluating additional safety and efficacy characteristics of the rabies vaccine for wildlife under limited field conditions at a specific site. The basis for this approval would include a finding of safety under 9 CFR 103.3, and would be supported by a "Finding of No Significant Impact" under 40 CFR 1500-1509.

VI. Affected Environment and Environmental Consequences

A. Site Description

The proposed field trial site is on Pennsylvania State Game Lands (SGL) #13 in Sullivan and Lycoming Counties. This area is in northeastern Pennsylvania adjacent to the village of Elk Grove and can be characterized as sparsely populated, broadly wooded, very hilly with intervening streams, and with elevations ranging from 350 to 750 m (see map, figure 1). The region is situated on the Appalachian Plateau and has an underlying rock strata of flat-lying sandstones and shales. Soils are typically poor in structure and mineral composition, consisting of acidic and well-drained shallow sandy and stony loams. The climate is characterized by severe winters and cool summers, with four to five frost-free months (May to September) and 10 to 20 cm precipitation per year. Dominant vegetation of the area is a mix of northern hardwood and coniferous forests, punctuated by an occasional highland glacial swamp.

Two core study areas will be utilized within SGL#13, designated the Shingle Mill and the Grassy Hollow sites. The Shingle Mill site is west of the village of Elk Grove and extends along Fishing Creek. The southern portion of this site lies in a riparian valley, where the dominant tree species are red maple (*Acer rubrum*) and young saplings of beech (*Fagus grandifolia*), with scattered black birch (*Betula lenta*), grey birch (*B. populifolia*), paper birch (*B. papyrifera*), witch hazel (*Hamamelis virginiana*), and small groups of hemlock (*Tsuga canadensis*). Blackberry (*Rubus allegheniensis*) and evergreen wood fern (*Dryopteris spinulosa*) occur in the understory. The northernmost portion of the site adjoins a south-facing slope that in some sections is very steep. Dominant tree species are again hemlock and red maple, with occasional sugar maple (*Acer saccharum*), scarlet oak (*Quercus coccinea*), white oak (*Q. alba*), chestnut oak (*Q. prinus*), red oak (*Q. rubra*), pin oak (*Q. palustris*) and beech. Ground cover in open sunny spots is dominated by the hay-scented fern (*Dennstaedtia punctilobula*), and club moss (*Lycopodium spp.*) occurs in the shadier areas.

The Grassy Hollow site, east of the Shingle Mill site and north of Jamison City, extends up a steep creek valley on an east-to-north facing slope, switchbacks up the slope (now west-facing), and crosses a portion of the high plateau. Dominant tree species on the rocky east-north slope are red maple, hemlock, black birch, and beech, with evergreen wood fern as ground cover.On the west-facing slope the only apparent change in plant composition is a replacement of the evergreen wood fern with the hay-scented fern. The trail through the Grassy Hollow site begins near Lewis Falls at an elevation of 500 m, and after a distance of 7 km has risen 200 m. It then rises 80 m to a plateau in a distance of 2 km. Some of the Grassy Hollow site is in or adjacent to a logged forest that is now cut-over slash containing

grasses and young beech. The forest edge of this slash area contains red maple and striped maple (*Acer pennsylvanicum*), with hay-scented fern, blackberries, grasses, or sphagnum moss (*Sphagnum spp.*) on the ground. Further into the forest are hemlock, red maple, and beech, with scattered scarlet oak, shagbark hickory (*Carya ovata*), and shellbark hickory (*C. laciniosa).*

In disturbed areas on both sites occur norway spruce *(Picea abies*), scotch pine (*Pinus sylvestris*), eastern white pine (*P. strobus*), and occasional larch (*Larix occidentalis*), aspen (*Populus grandidentata*), mountain ash (*Sorbus americana*), basswood (*Tilia americana*), yellow buckeye (*Aesculus octandra*), sycamore (*Plantanus occidentalis)*, and tulip poplar (*Liriodendron tulipifera*). In the understory occur dogwood (*Cornus florida*), sassafras (*Sassafras albidum*), laurel (*Kalmia latifolia*), wild grape (*Vitus aestivalis*), and rose (*Rosa multiflora*). Mammalian species occurring on the Shingle Mill and Grassy Hollow sites are discussed in section V.D.

B. Physical Environmental Risk

The V-RG vaccine, per se, is not expected to have a direct impact on the physical environment of SGL#13. Vaccine will not be released into the air, thus the inhalation of vaccine materials is unlikely. Vaccine-bait packages will be placed on the soil, and if damaged, the contents could contact the soil. These packages contain mostly bait—a highly biodegradable combination of organic constituents that should either be consumed rapidly by various organisms or chemically degraded upon exposure to light, heat, and/or water. The vaccine liquid and the associated capsule and fishmeal polymer substrate are also biodegradable and nontoxic. The vaccine-bait combination may also leach into the soil, or be carried by rainfall runoff to adjacent streams. The rapid utilization of this material by animals or degradation by other means, and the absence of toxic substances, indicates no threat to groundwater or surface waters.

During the previous field trial by Wistar of the V-RG vaccine on Parramore Island, Virginia, approximately 25 individual researchers were on site at some time during the first 2 weeks of vaccine-bait placement. At the proposed Pennsylvania site, this number of individuals walking or driving on site could have an impact on the environment. The SGL#13 site is composed of mostly sloping terrain with shallow and fragile soils. Trampling of vegetation and loose soil may lead to erosion and subsequent damage to both hillside and stream ecosystems. Smoking by project personnel and visitors, as well as the use of campfires, could pose a forest fire threat. Improper disposal of used materials may also produce litter or contamination. Vehicle use by project personnel and visitors may impact both the physical and biological environment. Exhaust fumes, airborne dust from the unpaved roads, and vehicular noise may all be factors impacting habitats and animals adjacent to roads. Mitigations developed by Wistar will reduce these potential impacts to insignificant levels (see section VI).

C. Human Health Risks

1. General Risk Factors

Potential human health risks associated with the field release of the V-RG vaccine are (1) infection with vaccinia from the vaccine, (2) infection with vaccinia or rabies from animals, and (3) infection with other diseases or injury from animals. The V-RG virus consists of a vaccinia virus that is functioning as a vector for one gene that codes for only one rabies virus surface glycoprotein, and not for any part of the infective rabies virion. Thus, a human that somehow might become infected with the V-RG virus could develop antibodies to the rabies surface glycoprotein but could not become infected with rabies. Although poxviruses do occur naturally in wildlife populations, vaccinia is not known to occur in the wild outside of vaccinated animals (see section V.E.1 for discussion of buffalopox). It is possible, however, that an animal that has recently consumed an oral vaccine containing vaccinia virus may have virus in the mouth that could be mechanically transmitted to another animal or human by bite. Prior vaccinia immunization and care in the handling of animals will minimize this risk to humans.

Rabies virus does occur naturally in wildlife and may be present at the proposed field trial site. Humans who will be handling animals as part of this project are at some risk if they are bitten. Prior rabies immunization and care in the handling of animals will minimize this risk.

2. Post-Infection (Vaccination) Complications

There are certain risks associated with vaccinia vaccines, including the V-RG proposed for wildlife vaccination and the New York Board of Health strain used in human vaccination in the United States. It is conceivable that a human could consume or otherwise contact some of a vaccine-bait package, or be bitten by an animal that had consumed vaccine-bait within the several previous days, and subsequently develop a vaccinia virus infection. As such, the individual would be at some risk of developing post-vaccinal complications. These complications have been broadly classified as either dermal in origin or involving the CNS (90). The principal CNS complication is usually postvaccinal encephalitis, with signs and symptoms of a generalized viral encephalitis. Vaccinia virus is not routinely isolated from the CNS lesions; the nature of the complications are thought rather to be allergic in nature. Mortality in post-vaccinal encephalitis cases usually ranges from 10 to 30%. Rates for post-vaccinal encephalitis (all ages) in the United States averaged 3 per million primary vaccinia virus vaccinees.

Examining the combined U.S data for all ages, the most prevalent (New York Board of Health strain) vaccinia-induced complications were dermal in nature, classified as vaccinia necrosum or progressive vaccinia (1 per million), eczema vaccinatum (40 per million), accidental infection (500 per million), generalized vaccinia (200 per million), and erythematous urticarial eruptions (unknown rate) (90). The first two complications were associated with immunological deficiency and atopic dermatitis, respectively. Vaccinia necrosum was characterized as progressive necrotic lesions originating from the inoculation site; vaccinia virus could be isolated from these lesions.

Eczema vaccinatum was less severe, with a syndrome resulting from local spread or vaccinia dissemination, clinically similar to Kaposi's varicelliform eruption. Accidental vaccinia virus infection, while not uncommon during the period of mass vaccination programs, was usually mild and self-limiting. These infections were usually due to autoinoculation from the primary vaccination site to other body areas (usually involving the mucus membranes), or were a result of intimate body contact between an unvaccinated individual and the active lesion of a recent vaccinee (23). True generalized vaccinia was a rare, nonspecific dermal complication in the nonatopic and immunocompetent patient, manifested as a vesicular rash which developed related to the act of vaccination. There was no true etiological basis for the rash, since detectable viremia and vaccinia virus could not be isolated from the multiple, small, erythematous vesicles which formed. The final dermal complication, erythematous urticarial eruptions, was observed within 7 to 12 days following vaccinia inoculation. No specific treatment was recommended, as for generalized vaccinia, because the pathogenesis was unknown. These patients remained afebrile, and the rashes were self-limiting after several days.

All of the above CNS and dermal complications in humans were associated with the act of intentional intradermal inoculation of high concentrations of parental vaccinia virus. Considering the low probabilities of these complications occurring in normal vaccination procedures, the attenuated nature of the V-RG recombinant vaccinia virus (see section II.C.2), the proposed restricted nature of its use in the field trial, and the containment of the virus in a bait, it is extremely unlikely that such vaccine-induced complications would occur in humans as a result of this field test.

3. Oncogenicity

The potential oncogenicity of the V-RG virus in humans is a parameter that should be considered. Vaccinia virus is not known to be a tumor-inducing virus. There have been no documented reports of oncogenicity associated with natural vaccinia virus infections in any animal species. The recombinant DNA methods used for the preparation of the V-RG recombinant virus do not introduce any known oncogenes into the already nontumorigenic vaccinia virus that could cause it to become tumorigenic. At least one report describes a vaccinia virus strain with reduced lytic properties capable of cell transformation *in vitro* (82), but there are no supportive data for oncogenicity of vaccinia virus *in vivo* despite its widespread human use in the smallpox eradication campaign.

4. Virulence for Immunodeficients

Humans with compromised immune systems are at greater risk to a wider variety of viral diseases than immunocompetent individuals. The virulence of the V-RG virus for immunocompromised animals was investigated by Wistar in several laboratory experiments. One study examined the response of athymic immune deficient nude mice when V-RG was inoculated intradermally into the tail. V-RG virus was recovered from internal organs (spleen, intestine, and adrenal gland) of mice killed on day 9, but was not recovered from mice killed on days 2, 4, 7, and 11. This suggests that there was a

V-RG viremia with subsequent infection of other organs in the body, although suspected viremic animals in this study appeared healthy at necropsy. In a companion study with immunocompetent mice, no suggestion of viremia was noted.

An additional study was conducted in athymic nude mice by Wistar. Thirty mice were inoculated on the dorsal tail surface with 40 ul of V-RG with a titer of 10^9 per ml. On days 5, 7, 9, 11, 13, and 15, five mice were euthanized and a complete necropsy was performed. Virus isolation was attempted on skin, lung, liver, kidney, spleen and adrenal gland. All mice remained clinically normal, though typical vaccinia lesions were observed at the injection site on all mice. Three of the five mice in the last group no longer had scabs at the injection site by the 15th day and the lesions were beginning to resolve. Virus isolation attempts in cell culture were positive for many of the organ homogenates on most of the sample days. No consistent pattern of virus isolation was present, although the titers of isolated virus declined as skin healing progressed. Pathology attributable to the V-RG virus was not detected in any organ, despite the presence of virus.

Although virus replication does occur in athymic nude mice, apparently the ability of other elements of the immune system to respond effectively to infection with V-RG virus is sufficient to prevent gross pathology, morbidity, and death. These experiments in mice suggest than immunocompromised humans, when accidentally exposed to V-RG virus, are at minimal risk (see appendix E for further details).

5. Reversion to Virulence or Recombination

An area of concern involving recombinant genetic products is the possibility of reversion to a previous state. Wistar has examined the genetic stability of the V-RG recombinant virus in laboratory experiments. A concentrated suspension of the vaccine (10^8 $TCID_{50}$/ml) was inoculated into 6-week-old CF1 mice, using the plantar pads (cushions) of the posterior feet (intraplantar method). After 48 hours, mice were euthanized, and the top ends of the feet were homogenized in a physiological solution. After clarification, the supernatant was reinoculated into other mice by the intraplantar method. After four serial subpassages of the V-RG recombinant vaccine virus by this method, virus was not isolated from the homogenized tissues using cell culture isolation techniques. In another experiment, 6-week-old CF1 mice were inoculated i.c. with $10^{7.8}$ $TCID_{50}$ of V-RG vaccine virus. The brains of mice that died 3 to 4 days following inoculation were collected, homogenized, and subpassaged a second time into mice by the i.c. route of inoculation. The inoculum from the second passage of virus did not cause mortalities. Mice were killed 6 days post-inoculation and brain material was collected and subpassaged a third and fourth time by i.c. inoculation. Virus was not recovered from the third and fourth blind passage through mice brains. These experiments demonstrate that the V-RG virus does not gain in virulence by repeated animal passage. If biological transmission of the V-RG virus to other wildlife or humans should somehow occur, there is no indication from these limited studies that reversion to virulence would occur.

The theoretical recombination event of V-RG vaccine virus with a naturally occurring poxvirus, and the subsequent generation of a true and stable recombination-derived vaccinia virus with a functional TK gene, was also considered. The chance of recombination in raccoons would depend not only upon the natural prevalence of raccoon pox (RP), but also on the highly unlikely simultaneous infection of the same animal by both RP and V-RG viruses and the dual infection of the same cell. RP is not prevalent in the environment, with only two concurrent isolations in the United States (50), and has not been isolated from raccoons at the proposed vaccine release site. The probability of a recombination of RP and V-RG is thus very low. Laboratory experiments conducted at Wistar also demonstrated the absence of interference phenomena or adverse effects when RP-infected mice were subsequently inoculated with V-RG vaccine virus (table 3).

The probability of recombination of the V-RG virus with naturally occurring poxvirus in other animals and consequent generation of a vaccinia virus possessing an intact TK gene is also very low (11). The chance of recombination between V-RG and various indigenous avipoxviruses, or other orthopoxviruses, will vary with the taxonomic relationship of the indigenous poxvirus to V-RG virus and the natural prevalence of each virus. If a relatively unrelated wild virus (e.g., a leporipoxvirus) is involved, the recombination probability of reconstructing the original TK gene sequences in the vaccinia vector should still be quite low. The combination of two leporipoxviruses to form a new entity is known, but the combination of a leporipoxvirus with an unrelated poxvirus is unknown (45). Conceivably, combination of V-RG virus with unrelated DNA viruses could occur, but would be extremely unlikely.

V-RG virus is eliminated from orally inoculated animals within 48 hours, requiring that any recombination between the vaccine virus and an indigenous virus must occur within this very restricted period of time, or that a latency factor or persistent infection must occur. Such a latent or chronic infection has not been found among nearly 100 captive raccoons inoculated with V-RG vaccine virus by Wistar and tested for virus presence by standard methods.As such, the probability in which animals are persistently infected with the V-RG equates to a putative order of magnitude less than 1%. A similar probability for persistent infection of less than 1% must also be postulated for RP infection in raccoons. Although a previous survey suggested a seroprevalence of RP as high as 20% (2), Wistar has not obtained any evidence of RP infection among raccoons on the basis of several hundred raccoon sera screened for anti-RP virus neutralizing antibody activity. Moreover, there have been only two isolations of RP virus, which occurred more than 20 years ago at a single geographical location (50), with no further reports throughout the United States, suggesting a prevalence less than 0.1% at present. Thus, the probability of simultaneous or superinfection with V-RG and RP in the same animal and genome contact in the same cell with regeneration of an intact TK gene, is on an order of magnitude of 10^{-8} or less.

6. Other Diseases or Injury From Wildlife

Wildlife in the study area may possess zoonotic diseases other than rabies that could cause diseases in personnel involved in the field trial. Rocky Mountain spotted fever, Lyme disease, and viral encephalitis may occur in local mammals and birds and be transmitted to humans by various arthropod vectors. Project personnel will be alerted to this possibility and warned to take precautions to minimize contact with ticks, chiggers, and mosquitoes.

Some animal species in the study area also may pose a direct threat to project personnel. Black bears, bobcats, and poisonous snakes have the potential to cause injury. Project personnel will wear appropriate attire on lower extremities to reduce the risk from snake bite, and will attempt to avoid contact with large carnivores. The risk associated with project activity in areas where these animals are present is not excessive compared to those risks encountered by anyone engaged in outdoor activities in the area.

D. Wildlife

1. Target Raccoon Populations

The raccoon (*Procyon lotor*) (Carnivora:Procyonidae) is an important game species and competitor for human food that is both widely distributed and abundant, and is a species that has been useful in the monitoring of zoonoses and environmental pollutants (72). The current mid-Atlantic rabies epizootic is primarily associated with the raccoon, and considerable research has been conducted on this species both in Pennsylvania and at other locations.

a. Background Biology

The raccoon is distributed in wooded and brushy areas near water from the Gulf of St. Lawrence west to Southeastern British Columbia, south throughout the United States, Mexico, Central America, and into South America (93). Head and body length of mature animals ranges from 415 to 600 mm, with males usually larger than females, and with body weights ranging from 6 to 11 kg for adults from the northern part of its distribution (92). Most activity occurs at night; with denning during the day in trees, ground cavities, or structures (131). At northern latitudes comparable to SGL#13, raccoons in winter do not hibernate but may remain inactive in a den for most of the season and emerge only during intervals of warm weather (92). Raccoons possess very omnivorous food habits, consuming arthropods, aquatic animals such as frogs, crayfish, and fish, terrestrial small animals, bird eggs and nestlings, and a variety of plant material including fruits, berries, and corn (49). Social organization is probably polygynous, with territoriality apparently occurring only in adult males as a result of competition for females (43). Mating occurs in late winter and early spring; and after a gestation perod of 63 days, a litter of2 to 6 pups is usually born in the April to June period (112). Juvenile males may reproduce their first winter, whereas juvenile females may not initiate breeding until their second winter (112). Mortality rates associated with such factors as starvation, canine

distemper, listeriosis, and rabies may be high, with complete population turnover in 3 to 5 years (72).

b. Site Population

Trapping of raccoons began on SGL#13 in the fall of 1989 and continues to the present time. Captured animals were either sacrificed for post-mortem examination or released after data collection. All released animals were ear-tagged with a unique number, and some were additionally fitted with a radiotelemetry collar. Considerable effort was required to capture raccoons, as trap success was usually below 10% and in winter below 1%.

Based on trapping and mark-release-recapture data, the estimated 1990 summer population for the Shingle Mill and Grassy Hollow study areas combined was 1 raccoon per 5.0-6.7 ha (15-20/Km^2) (Peterson-Lincoln Index), assuming a reproductive rate of two to three surviving offspring per lactating female. Population density estimates in the literature range from 1 animal per 200 ha (43) to 1 per 0.5 ha (68), with most estimates in the range of 1 animal per6 to 18 ha. These estimates from SGL#13 are similar to values from other woodland sites (e.g., 83), and suggest that the SGL#13 raccoon population is representative of such sites.

Use of radiotelemetry enabled several unique types of data to be collected from raccoons. During 1989-90, 12 raccoons were captured and fitted with radio collars capable of transmitting in both activity and mortality modes. These transmitters had a lifespan of approximately 10 months and have all failed. Five animals in 1991 have received new collars and are currently being monitored, with additional animals to be collared. Each month, more frequently during spring-summer-fall, animals were monitored by telemetry receivers, with locations determined by triangulation of telemetry signals and plotting on U.S.G.S. topographical maps. Analysis of this data will not be completed until the radio collars placed on 1991 animals have expired. Patterns of raccoon population structure, density, and habitat use, documented on SGL#13 prior to the release of V-RG vaccine, will be invaluable in documenting the presence or absence of subsequent changes related to the V-RG vaccine release. Preliminary review of the present data, however, indicates patterns similar to those obtained from studies conducted at nearby locations (49,80).

A Pennsylvania Game Commission study (49) at Butler Hill during 1986 with 9 radio-collared raccoons documented an average spring home range of 47 ha, 48 ha during summer, and 18 ha during late fall and winter. Average home range of individual animals for an entire year was 66 ha, with some overlap of adjacent home ranges and no abandonment. Two raccoons left the riparian valley during the year, and these animals briefly moved over the ridge into the adjacent valley and then returned. Bottomland along the stream was the major area of activity throughout the year, where frogs, berries, and corn were the principal foods. Another study on SGL#211 (80) during 1987-88 with 10 radio-collared raccoons documented an average

annual home range size of 363 ha. Considerable overlap of home ranges occurred, particularly in late summer and fall, and was postulated to be necessitated by the topography of the riparian valley system. Two of the 10 monitored raccoons dispersed in the autumn out of the valley by traveling a distance of 2.1 km over the mountain ridge into an adjacent valley. The greatest straight-line distance traveled within any 24-hour period by an individual animal was 1.9 km.

Several studies have documented substantial late summer to fall dispersal of raccoons, particularly juvenile males (43,131). Mark-release-recapture and radiotelemetry studies in the Ridge and Valley area of Pennsylvania, however, have documented that the vast majority of raccoon activity within a valley population is restricted to that valley. There is thus a low probability that raccoons on SGL#13, exposed to V-RG vaccine, will move from their home stream valley to other areas.

2. Nontarget Populations

Beginning in the autumn of 1989, the mammalian population of SGL#13 was monitored on a routine basis by trapping, carcass identification, direct observation, and by such animal sign as tracks, scat, and feeding damage (table 5). Bird, herptile, and fish populations were observed, but systematic monitoring was not implemented due to the lack of involvement of these vertebrates in rabies epizootics. The mammalian fauna of the proposed field trial site can be characterized as typical of a northern temperate forest under minimal harvesting pressure. The most abundant animals were the small-sized mice, lemmings, voles, moles, and shrews. The oppossum, raccoon, and porcupine were the most abundant mid-sized animals, with the white-tailed deer and the black bear occasional inhabitants. Potential nonraccoon reservoirs of rabies virus in this mammalian population, such as red fox and striped skunk, were in low densities relative to the raccoon and probably would be minimally involved in any future rabies epizootic in the area.

3. Threatened and Endangered Species

There are no federal or state threatened or endangered vertebrate species listed for Sullivan and Lycoming Counties, Pennsylvania, based on listings in the Federal Register and a listing compiled by the Pennsylvania Game Commission on October 15, 1990.

4. Potential of V-RG to Infect Vertebrates

The potential of the V-RG virus to infect target and non-target species was evaluated in laboratory studies. More than 40 vertebrate species were inoculated by a variety of routes, including oral, and the subsequent development of antibodies monitored. All mammalian species that were tested became infected with the V-RG virus, developed antibodies to V-RG, and did not show detrimental effects from the immunization. Avian species developed very low antibody titers, with many individuals apparently refractory to the V-RG virus (see section III.B.1.).

The V-RG vaccine is not stable indefinitely under field conditions (see section V.E.1.a.). To determine the effect of inactivated V-RG vaccine on safety and efficacy, experiments were conducted in outbred and inbred

strains of laboratory mice using both live and inactivated vaccine (143). Vaccination was performed by inoculation into the footpad and by i.d. scarification of tail skin with parental vaccinia or V-RG virus (10^9 pfu/ml). Inoculation of mice with V-RG virus resulted in a rapid induction of rabies VNA titers of 30,000 after 14 days. All mice immunized with V-RG virus resisted challenge on day 14 with street rabies virus by i.c. inoculation of 2,400 mouse LD_{50}. Mice immunized with parental Copenhagen vaccinia virus were not protected when challenged. V-RG virus inactivated by betapropiolactone was inoculated i.p. on days 0 and 7, and mice were challenged with 240 mouse LD_{50} of lethal rabies virus on day 14. The inactivated preparation induced sufficient levels of rabies VNA to protect the mice against rabies challenge and was not associated with adverse host effects.

The effect of multiple inoculations of the V-RG virus on immune responses was evaluated in the laboratory. Three rabbits immunized i.d. with $10^{7.6}$ pfu of V-RG virus and showing 15 days after vaccination a VNA titer greater than 30,000, were inoculated i.d. 6 months later with the same dose of virus. Following the booster inoculation, the levels of VNA in all 3 animals increased dramatically starting on day 3 (titers 24,000) and reached titers of 70,000 or higher by day 15. Twenty-one days after the booster, the rabbits were challenged i.c. with 24,000 mouse LD_{50} of street rabies virus. All animals resisted challenge with street rabies virus and were free of adverse effects that could be associated with V-RG vaccination. These results indicate that the initial immunity induced by V-RG virus did not interfere with infection and antibody development associated with subsequent vaccination with the V-RG virus (142) (see section V.D.5.).

The red fox (*Vulpes vulpes*) and the striped skunk (*Mephitis mephitis*) are major wildlife reservoirs of rabies in some parts of the United States and Canada, and although not the target animals in the proposed Pennsylvania field trial, are present at this site and have been extensively tested for safety and efficacy with the V-RG vaccine. In laboratory experiments conducted by Wistar, 47 adult and juvenile foxes were inoculated with the V-RG vaccine via the oral route, and then challenged. All foxes were subsequently free of local or general reactions that could be associated with the V-RG vaccine, developed antibodies, and were protected against rabies by all but the lowest titer of vaccine. The V-RG virus was infective and confered some protection against rabies even when inoculated by nonstandard routes, such as the eye or nose. Entry by nonstandard routes did not alter the vaccine's safety.

Skunks were vaccinated with V-RG by oral administration in sponge baits and by deposition into the duodenum with an endoscope (130). Most animals developed detectable antibodies by 14 days post-vaccination. Over the 3-month observation period, titers decreased in all groups and all animals remained healthy. Vaccinated and control skunks were challenged i.m. with a suspension of salivary glands from naturally infected skunks ($10^{6.3}$ mouse intracerebral LD_{50}) and observed for 90 days. Five of eight

skunks vaccinated by eating bait survived the challenge, as did four of eight skunks in the intestinal group. All animals in the nonvaccination control group developed rabies between the 3rd and 4th week after challenge and died. At no time in the studies did the skunks which were fed V-RG bait appear unhealthy; no lesions were detected by gross or histopathological examination of the alimentary tract or other visceral organs (130).

5. Effect of Overdosage in Target and Nontarget Species

There is no evidence of potential harm to target or non-target species arising from overdosage of V-RG vaccine by any route or from multiple doses. The major rabies vector species (raccoons, skunks, and foxes) have each received in excess of a 10-fold field dose numerous times in the laboratory without ill effects. Non-target species such as gulls, rodents, deer, and coyotes have received orally 2 to 10 times the field dosage without adverse effects. In fact, rabbits demonstrated a typical dose response or anamnestic response to multiple doses and were better protected against subsequent challenge (table 4) (see appendix C for further details).

Considering baiting density, animal distribution patterns, and the rather limited mobility of vertebrate consumers, it is unlikely that any individual animal will contact more than two to three vaccine-laden baits over the relatively short baiting interval in the field. Moreover, multiple bait consumption studies (with or without vaccine) by target and nontarget species in the laboratory have not resulted in any ill effects. It is thus unlikely that the ingestion of numerous baits in the field would indirectly produce nonspecific effects on health, such as impaction or GI obstruction.

6. Multiplication, Transmission, and Dispersal of V-RG

The V-RG virus multiplies to a very limited degree in host tissues, and occurs in only a few tissues associated with the oral cavity after oral inoculation. After ingestion of bait, V-RG virus is not detectable in animal tissues for more than 48 hours. Transmission by laboratory passage (i.e., by tissue homogenization and inoculation of organ extracts) is limited to two or three passages. The likelihood of direct biological transmission via viral shedding has been largely dismissed by results of contact experiments with a variety of animals. Raccoons not directly inoculated or fed V-RG, but held in the same containment room with vaccinated animals, remained uninfected and fully susceptible to virulent street rabies challenge. Contact transmission has occurred in the laboratory among pair-bonded raccoons of the opposite sex, or between lactating females and their young, when an orally inoculated animal was immediately placed in contact with a nonvaccinate. The likelihood of such transmission occurring under natural conditions is considerably reduced(see section III.B.1 and appendix C for further details).

With biological and mechanical transmission of V-RG by vaccinated animals unlikely, the possible range of dispersal of the V-RG recombinant virus would be limited to the distance a bait might be carried by an animal or by arthropod transmission. V-RG virus does not circulate systemically in immunocompetent mammalian hosts; thus haematophagous arthropod involvement in transmission of V-RG is not expected. To support this suggestion, no arthropod vectors for vaccinia have been described. Observations at

bait sites during placebo bait trials and during previous V-RG field trials indicate that vaccine-bait units are usually consumed at the site of initial placement. When vaccine-bait units are moved, it is usually because the potential consumer is disturbed by another animal. Consumption then occurs within 10 feet of the initial placement site. Transport of vaccine-bait units by adult female raccoons to their dens for feeding to juveniles has not been observed. Dispersal of the vaccine-bait units by scavengers or other nontarget animals is not expected to be a significant factor.

E. Cumulative Impacts

1. Persistence in Environment

a. V-RG Vaccine

Rabies virus is not known to persist outside animal hosts. Replication and spread is dependent on infection of new susceptible hosts. Quick freezing followed by dessication preserves the virus, but slow drying typical of field situations is detrimental to the virus, as is exposure to ultraviolet radiation. Infection of new hosts is generally through bite wounds, though aerosol transmission in at least three human cases has been recorded (1). These were associated with bat caves or occurred as a result of laboratory accidents. Except for these few cases, there is no evidence that rabies can be spread other than through direct contact with an infected animal. Since the V-RG vaccine, however, is only capable of eliciting host production of a rabies surface glycoprotein and not the infective rabies virion, there is no chance of accidental introduction or persistence of rabies from this vaccine.

V-RG virus has been isolated in raccoons only from host tissues associated with the mouth after oral inoculation, and only in the first 48 hours post-inoculation. A viremia does not occur, and lesions in other organs are not found. Thus a raccoon carcass, though it may be persistent, is not likely to be a source of V-RG virus to potential consumers.

Extracellular persistence and stability of the V-RG virus is highly dependent on ambient temperature and local environmental conditions. Results from titration experiments performed by Wistar show that V-RG vaccine, in both the lyophilized and reconstituted liquid forms, is stable at and below 4°C with no appreciable loss of titer. At temperatures between 20° and 37°C (which mimic summer field ambient temperatures) liquid viral vaccine titer remains stable for approximately 14 and 7 days, respectively, in both the original sterile vaccine ampule or inside a bait. Minimal loss of titer is thus expected within baits under climatic conditions of the proposed field trial sites during spring and early summer. Infective titers should be maintained for at least 2 to 3 weeks, with rapid inactivation occurring over the next several weeks. The V-RG virus loses greater than 10^7 log of infectivity when allowed to dry upon moist sand within 7 days at 35°C and 21-28 days at 25°C. The V-RG virus also loses most of its infectivity in fresh water within 21-28 days at 4°C, and within 7-14 days at 35°C. If V-RG virus is allowed to

dry upon vegetation, greater than 10^7 logs of inactivation occurs within 7 days at 35° to 14 days at 25°C.

b. Vaccinia

Vaccinia virus is susceptible to inactivation by alkaline or acidic pH, and by bile, lipases, certain fatty acids, and numerous oxidizing agents (52,132). Due to V-RG gastrointestinal inactivation and rapid clearance when consumed orally, environmental contamination with vaccinia via feces should be minimal. A more likely method of contamination would involve actual vaccine spillage during the act of bait consumption or continued presence of incompletely consumed vaccine-bait. Vaccinia virus under normal environmental conditions may persist for prolonged periods outside the host's body if protected from sunlight. The virus withstands drying, and may persist for years if properly desiccated and maintained at refrigerator temperatures. Environmental contamination, however, has never been implicated in the persistence and transmission of vaccinia virus (14), suggesting that the V-RG virus will also be adversely affected by environmental parameters. The attenuation of vaccinia in the V-RG vaccine through the disruption of the TK region further reduces the possibility of persistence in the environment, as does the limited period (10 days) of vaccine-bait placement in the field trial.

c. Buffalopox

A 1989 publication (38) provided evidence that buffalopox virus isolated from animals in India was very similar to vaccinia virus. This brought into question the assumption expressed in the Environmental Assessment published on March 3, 1989, that no vaccinia virus was known to exist in nature. In 1985 and 1986, scab material was collected from buffalo in Maharashtra State in India, resulting in the isolation of 13 pox viruses. Of these, 12 were typical of buffalopox virus, but 1 was indistinguishable from vaccinia in genome structure and biological characterization. In India prior to 1978, vaccinia virus had been grown in the skin of buffalo calves to produce smallpox vaccine. At some point in vaccine production, a change in the virus may have occurred, resulting in the emergence of a buffalo-adapted strain. Human vaccination was subsequently discontinued in 1979, and it was assumed that with the cessation of smallpox vaccine production, lesions in buffalo would disappear. Although buffalopox has previously been reported from Egypt, Bangladesh, and Indonesia, there is no current information on sustained infection in those countries. The persistence of buffalopox and the isolation in India of an apparent vaccinia virus 6 to 8 years after cessation of vaccinia production indicates that a natural cycle of virus transmission may have developed. Since that time, studies have determined that buffalopox virus is distinct from vaccinia but may be considered a subspecies of vaccinia (Dumbell 1990, unpubl.).

The production of smallpox vaccine in bovines and buffalo relied on the ability of the vaccinia virus to grow to high titers on the skin of these

animals. The V-RG vaccine to be used in the proposed Pennsylvania field trial has been shown not to reproduce to titers high enough for natural spread in any species, including cattle. V-RG has been tested in representative species of all mammal groups known to occur in the field trial area, and none of these have supported replication of the virus when administered orally.

2. Effects on Wildlife Populations

Laboratory investigations and previous field trials have demonstrated conclusively that the V-RG vaccine will have no direct and immediate effects on target or nontarget populations. Indirect or long-term effects of the V-RG vaccine are not so easily discounted.

Effects of trapping, handling, and release of animals in the field trial area on their future health, behavior, and population levels are difficult to predict. Based on other field studies, however, those effects are not expected to be significant provided that standard procedures for such activities are followed. The impact on target animal populations of accidental mortality associated with trapping and handling, and programmed mortality associated with histopathology sampling, is also considered not to be significant given the relatively low mortality anticipated.

Another factor that could have a long-term cumulative impact on animal populations in the area of the field release is the incidence of rabies in raccoons. After several years of sampling on SGL#13, the first raccoon with detectable rabies antibody was discovered in 1990. This occurrence suggests that the advancing front of the mid-Atlantic rabies epizootic has reached Sullivan County, Pennsylvania. Increased mortality due to rabies would be expected in the raccoon population over the next several years, leading either to eventual stabilization at lower population densities or to development of compensation mechanisms such as increased reproductive rates that would maintain populations at current levels. The application of the V-RG rabies vaccine at this early stage in the local epizootic may eliminate these possible scenarios and allow the raccoon population to remain at present densities.

VII. Mitigative Measures

A. Worker Safety

Local medical personnel will be notified in advance of the vaccine field trial, so that a rapid response is ensured in the unlikely case of a medical emergency or the development of unusual lesions or illness in project personnel. Wistar personnel working at the study site will be immunized against vaccinia in compliance with U.S. Public Health Service guidelines for individuals working with recombinant vaccinia viruses. These personnel will also be vaccinated against rabies. State and federal personnel from cooperating agencies who will be actively involved in the field operations will be encouraged to obtain vaccinia and rabies vaccinations, kept fully informed about field trial activities, and cautioned about contact with baits containing V-RG vaccine.

B. Visitor Safety

Federal, state, and local observers will be permitted on the study areas, provided approval has been given. Though these individuals will be at minimum risk, they will be encouraged to obtain the appropriate vaccinations and will be cautioned about contact with vaccine-bait packages.

The probability of contact with the vaccine-bait for visitors to the study site will be minimized by characteristics of the baits and the site. The baits should be repellent and unattractive to humans due to their malodorous nature; warning labels on the bait packets should also reduce human contact. The isolated nature of the study sites, the anticipated pre-field trial publicity, and the short time the baits will remain in the field should further limit human contact.

C. Handling Of Vaccine

Vaccine materials in the Wistar laboratories will be handled under etiological agent category two bio-containment conditions. Safe laboratory practices formulated by Wistar will also be followed (Wistar Laboratory Safety Manual, 1988). Labeling, packaging, and shipping of vaccine-bait units will conform to current U.S. Public Health Service recommendations for handling of etiological agents. Laboratory and field work with the vaccine will be conducted by trained, experienced, and appropriately pre-immunized personnel.

D. Processing Of Animals

During V-RG field trials, live-trapped animals will be handled by trained, experienced, and appropriately pre-immunized personnel. Animals will be handled in such a way as to minimize injury to themselves and other

animals, as well as to project personnel. This will include appropriate sedation of animals and the wearing of protective equipment by personnel.

E. Restrictions On Site Access

The proposed vaccine-bait and surveillance areas within SGL#13 will be marked and posted on the periphery and at entrance sites with signs indicating that a oral wildlife vaccine trial is in progress and that access is restricted. During the initial 2 weeks of vaccine-bait placement, gates controlling road access to the study site will be closed and only authorized personnel will be allowed entrance. Access to the general area will also be monitored during this period by routine daily patrols of the periphery of the study area by at least one full-time person. Subsequently, warning signs will be left up until the end of the 12-month surveillance period, but monitoring of access to the study area will be discontinued after removal of the vaccine-baits.

F. Minimizing Impact On Environment Caused By Physical Presence And Activity Of Project Personnel

Project personnel will minimize their impact on the study site by eating in designated areas, sleeping at off-site housing or campgrounds, properly disposing of waste, not smoking on site, and parking vehicles in designated sites. Whenever possible, walking trails through bait and surveillance areas will be situated to minimize damage to vegetation and soil. The number of individuals walking these trails and the frequency of passage will also be minimized. Vehicle permits will be required from the State Game Commission, and driving in SGL#13 will be restricted to designated roads. Vehicular traffic by project personnel will be limited to that necessary to accomplish project objectives.

VIII. Monitoring

A. General

Bi-monthly progress reports, beginning 2 weeks after vaccine-bait placement, will be submitted to USDA, the Commonwealth of Pennsylvania, and other interested parties. At the end of 12 months, a public meeting will be convened for formal presentation and discussion of the field trial results.

B. Human

To monitor the potential exposure of project personnel to vaccinia and rabies virus, blood samples will be obtained from each individual 2 weeks before the start of the field trial, and at 1, 6, and 12 months after vaccine-bait placement. Sera will be tested for the presence of neutralizing antibodies to these viruses, and individuals seroconverting will be examined further.

For resident local populations, the Pennsylvania Department of Health (PDH) will serve as the coordinator of information concerning possible occurrence of vaccinia virus infections. Local medical personnel will notify the PDH of such infections, as will Wistar. The PDH will then notify federal authorities and other concerned parties and handle follow-up investigations.

C. Animal

In the unlikely event that illnesses or lesions attributable to the vaccine are discovered in humans, domestic animals, or wildlife, state officials will be notified immediately. Surveillance will be extended beyond the planned 12-month time frame and the area surveyed may be expanded. As indicated in the protocol section (II.A.3.c), monitoring of the raccoon and nontarget populations during vaccine-bait exposure will be daily. After vaccine-bait removal, monitoring will continue at 2-week intervals for 12 months.

IX. Glossary

APHIS	The Animal and Plant Health Inspection Service, U. S. Department of Agriculture
cDNA	Complementary DNA
CEF	Chicken embryo fibroblast
CFR	Code of Federal Regulations
CNS	Central nervous system
CVS	Challenge Virus Standard, a strain of rabies virus used for challenge in the National Institutes of Health (NIH) potency tests
DNA	Deoxyribonucleic acid
EA	Environmental Assessment
ERA	A trade name for a rabies vaccine derived from the SAD strain
Fixed rabies	A rabies virus which has been laboratory adapted, possessing relatively predictable and stable biological attributes
FONSI	Finding of No Significant Impact
HEP	High egg passage - a rabies vaccine modified by serial passage through more than 182 chicken embryos
ha	Hectare, a land measure of 100 meters squared (2.47 acres)
i.c.	Intracerebral or in brain tissue (a route of inoculation or location of infection)

i.d.	Intradermal or within the skin (a route of inoculation)
i.m.	Intramuscular (a route of inoculation)
i.p.	Intraperitoneal (a route of inoculation)
IU	International Units
LD_{50}	The dose which is lethal for 50% of the experimental animals
LEP	Low egg passage - a rabies vaccine modified by serial passage 40 to 50 times in chicken embryos
Mabs	Monoclonal antibodies
mRNA	Messenger RNA
Mouse LD_{50}	The amount of virus that is lethal in 50 percent of the mice infected; therefore 2,400 mouse LD50 is 2,400 times that amount of virus
NIH	National Institutes of Health, U.S. Department of Health and Human Services
PAHO	Pan American Health Organization
PHS	U.S. Public Health Service, U.S. Department of Health and Human Services
PFU	Plaque forming units, or pock forming units. These are measures of virus titer.
RFFIT	Rapid Fluorescent Focus Inhibition Test, a commonly used laboratory test for measuring antibody to rabies virus
RNA	Ribonucleic acid

RP	Raccoon Pox
SAD	Street Alabama Dufferin - a strain of rabies virus that has been used to develop modified live virus rabies vaccines
SDM	Sulfadimethoxine - a veterinary pharmaceutical used in the reatment of gastrointestinal disorders. Administered orally, it rapidly appears in the circulatory system and subsequently can be detected in blood sera for several days.
Street rabies	Field (or unmodified) strains of rabies virus
Sylvatic rabies	Natural rabies virus infection maintained in free-roaming wildlife
$TCID_{50}$	The concentration of virus that causes infection in 50 percent of tissue culture cells
TK	Thymidine kinase, an enzyme involved in the metabolic pathway of DNA synthesis and present in many cell systems of mammal
Trap-night	A measure of trapping effort; number of traps x number of nights; trap(s) in operation
VNA	Virus neutralizing antibody
V-RG	The Wistar Institute's vaccinia-vectored rabies vaccine created by inserting a rabies virus coat glycoprotein gene into the genome of vaccinia virus
WHO	World Health Organization
Wistar	The Wistar Institute of Anatomy and Biology of Philadelphia, Pennsylvania

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XI. Tables and Figure

Table 1. Protective immunity in mice from V-RG vaccine: effect of challenge virus and duration post-vaccination*

Challenge virus	V-RG vaccine concentration (log_{10})	Protection
Duvenhage	5.7	8/10
	5.0	8/10
	4.3	6/10
Controls	–	0/10
CVS-24	5.7	9/10
	5.0	9/10
	4.3	9/10
Controls	–	0/10

* 4- to 6-week-old female ICR mice were inoculated i.m. with 0.1 ml of V-RG on days 0 and 7 and challenged either on day 14 with 0.03 ml of Duvenhage virus i.c. (10^5 $MICLD_{50}$) or at 16 weeks with 0.1 ml of CVS virus i.m. ($10^{6.2}MICLD_{50}$).

Table 2. Intraspecific contact trials in raccoons*

Pair	Sex	Vaccine status	VNA		Challenge results
			Day 0	Day 30	
1	M	NI	<10	<10	D
	M	I	<10	3,645	S
2	M	NI	<10	15	S
	F	I	<10	135	S
3	M	NI	<10	<10	D
	M	I	<10	45	S
4	M	NI	<10	<10	D
	M	I	<10	70	S
5	F	NI	<10	15	S
	M	I	<10	3645	S

* Raccoons were housed together 30 days prior to one member of the pair receiving orally 2.0 ml of V-RG (10^8 pfu/ml) and challenged 30 days later i.m. with $10^{5.5}$ $MICLD_{50}$ of rabies virus strain MD5951; NI = not immunized, I = immunized; D = died, S = survived.

Table 3. VNA response in mice inoculated with raccoon pox and V-RG

Groups*	Day 0	Day 30	Day 60
Raccoon pox + V-RG	≤0.1	≤0.1	3.0 (1.0-10.0)
V-RG only	≤0.1	≤0.1	3.2 (2.0-18.0)

* ICR mice were inoculated IM on days 0 and 14 with $10^{4.5}$ TCID of raccoon pox virus or PBS and subsequently inoculated IM with $10^{6.4}$ pfu of V-RG 30 days later.

Table 4. Booster response* of rabbits to V-RG virus

Animal #	Rabies VNA titers				Protection
	Day 180	Day 183	Day 185	Day 195	
1	8,000	24,000	70,000	>70,000	+
2	8,000	24,000	70,000	>70,000	+
3	12,000	24,000	>70,000	>70,000	+

* Primary incoulation occurred on day 0 using $10^{7.6}$ PFU of virus i.d., with a booster (same dose and route) at 6 months. I.c. challenge with 24,000 $MICLD_{50}$ of rabies strain MD5951 occurred within 21 days.

Table 5. Mammals of State Game Land #13*

[Prepared by Wistar Institute]

Taxon	Common name	Abundance**
Marsupialia		
Didelphidae		
Didelphis virginiana	Opossum	0.6
Insectivora		
Sorcidae		
Sorex cinerus	Masked shrew	0.08
S. fumeus	Smoky shrew	
S. dispar	Longtail shrew	
S. palustris	Northern water shrew	
Cryptotitis parva	Least shrew	
Blarina brevicauda	Northern shorttail shrew	3.4
Talpidae		
Scalopus aquaticus	Eastern mole	
Parascalopus breweri	Hairy tail mole	
Condylura cristata	Star nose mole	
Carnivora		
Ursidae		
Ursus americanus	Black bear	
Procyonidae		
Procyon lotor	Raccoon	6.4
Mustelidae		
Mustela erminea	Shorttail weasel	
M. rixosa	Least weasel	
M. frenata	Longtail weasel	
M. vision	Mink	
Lutra canadensis	River otter	
Mephitis mephitis	Striped skunk	0.09
Canidae		
Canis familiaris	Dog	
C. latrans	Coyote	
Vulpes vulpes	Red fox	
Urocyon cinereoargenteus	Gray fox	
Felidae		
Felix domesticus	House cat	
Lynx rufus	Bobcat	
Lagomorpha		
Leporidae		
Lepus americanus	Snowshoe hare	
Sylvilagus floridanus	Eastern cottontail	
S. transitionalis	New England cottontail	

Table 5. Mammals of State Game Land #13* — continued

[Prepared by Wistar Institute]

Taxon	Common name	Abundance**
Artiodactyla		
Ceridae		
Odocoileus virginianus	Whitetail deer	
Rodentia		
Sciuridae		
Marmota monax	Woodchuck	
Tamias striatus	Eastern chipmunk	0.2
Sciurus carolinensis	Eastern gray squirrel	
Tamiasciurus hudsonicus	Red squirrel	
Glaucomys volans	Southern flying squirrel	
G. sabrinus	Northern flying squirrel	
Castoridae		
Castor canadensis	Beaver	
Cricetidae		
Peromyscus leucopus	White-footed mouse	0.8
P. maniculatus	Deer mouse	10.2
Neotoma floridana	Eastern wood rat	
Synaptomys cooperi	Southern bog lemming	
Clethrionomys gapperi	Boreal red-backed vole	2.9
Microtus pennsylvanicus	Meadow vole	0.5
Pitymys pinetorum	Pine vole	
Ondatra zibethica	Muskrat	
Muridae		
Rattus norvegicus	Norway rat	
Mus musculus	House mouse	
Zapodidae		
Zapus hudsonicus	Meadow jumping mouse	0.2
Napaeozapus insignis	Woodland jumping mouse	1.8
Erethizontidae		
Erethizon dorsatum	Porcupine	0.9

* Species known or thought to occur in the area based upon live-trapping, direct observation, carcass identification, animal sign, range maps, and habitat associations.

** Relative abundance approximated by number trapped per trapnight, multiplied by 100.


Wistar Rabies Vaccine
Surveillance & Vaccination Areas
PENNSYLVANIA
Legend
Vaccination
Surveillance
Scale
0 1 2
kilometers
contour interval
20 meters
STATE GAME LANDS NO 13
SULLIVAN CO
COLUMBIA CO
LUZERNE CO
LYCOMING CO
RICKETTS GLEN STATE PARK
SUGARLOAF
JACKSON
JORDAN
North Mountain
Ganoga Lake
Jamison City
Central
Grassmere Park
Nordmont
Long Brook
Sonestown
Muncy Valley
Hemlock Grove
Lake Jean
Red Rock
Fairmount Springs
Richarts Grove
Biggerstown
41°25'
41°20'
41°15'
76°20'
76°25'
76°30'

XII. Consultation And Review

A. Because of considerable public interest and concern about rabies virus, a concerted effort has been made by Wistar Institute and cooperating agencies to disseminate information about the proposed rabies vaccine field release trials. Wistar has made, and will continue to make available, detailed background information for distribution to local radio stations and newspapers.

In collaboration with the USDA and state agriculture, health, and game officials, Wistar personnel participated in a local public meeting in Sullivan County on October 24, 1990, to explain the proposed field trials and answer questions. A state-wide public meeting for the same purpose was conducted in Harrisburg, Pennsylvania, on November 14, 1990. Additional opportunities for public imput will be provided after the availability of this draft EA is announced in the Federal Register, both by written comment and by attendance at a public hearing scheduled for May 17, 1991, in Harrisburg, Pennsylvania.

B. The Wistar Institute, in preparing data to support its request to conduct open field trials with an experimental live genetically engineered rabies vaccine, consulted and developed information in cooperation with the following:

Agriculture Canada, Nepean, Ontario (Drs. K. Charlton, A. Wandeler).

Allegheny County Department of Health, Pittsburgh, PA (Dr. I. Chaudry).

Centers for Disease Control, Atlanta, GA (Drs. G. Baer, J. Esposito, M. Fekadu, D. Fishbein).

Cornell University, College of Veterinary Medicine, Diagnostic Laboratory, Ithaca, NY (Dr. D. Lien).

CSIRO, Victoria, Australia (Drs. M. Andrew, D. Boyle, B. Coupar, K. Fahey).

Commonwealth Serum Laboratories, Parkville, Victoria, Australia (Dr. R. Macfarlan).

Delaware State Department of Health and Public Services, Dover, DE (Dr. G. Spence).

Florida State Game and Freshwater Fish Commission, Gainesville, FL (Dr. M. Rolke).

Indiana University of Pennsylvania, Biology Department, Indiana, PA (Dr. R. Lord).

Johns Hopkins University, Department of Epidemiology, Baltimore, MD (Drs. J. Childs, H. Fischmann).

Medical School Observatory, Cape Town, South Africa (Dr. K. Dumbell).

Monel Institute, Philadelphia, PA (Dr. R. Mason).

National Audubon Society, Washington, DC (Ms. M. Hinkle).

National Wildlife Federation, Washington, DC (Dr. J. Rissler).

New Jersey State Department of Fish, Game and Wildlife, Hampton, NJ (Dr. D. Roscoe).

New Jersey State Department of Veterinary Public Health, Trenton, NJ (Dr. F. Sorhage).

New Jersey State Veterinary Medical Association, Springfield, NJ (Dr. R. Alampi).

New York State Department of Agriculture & Markets, Albany, NY (Dr. J. Huntley; Mr. J. Burnes).

New York State Department of Environmental Conservation, Albany, NY (Messrs. P. Martin, J. Lynch, R. Stumvoli).

New York State Department of Health, Clinical Laboratories, Albany, NY (Drs. L. Grady, J.G. Debbie, S.C. Frantz, L. Sturman; Mr. C. Trimarchi).

New York State Office of Parks, Recreation and Historic Preservation, Albany, NY (Mr. T. Lyons).

New York State Veterinary Medical Society, Westport, NY (Dr. R. Lopez).

New York State Wildlife Rehabilitation Council, Tully, NY (Ms. R. Borzik, Ms. D. Tessaglia).

Ohio State Veterinary Medical Association, Columbus, OH (Dr. K. Smith).

Ontario Ministry of Natural Resources, Maple, Canada (Drs. C. MacInnes, R. Rosatte; Messrs. D. Johnston, P. Bachman).

Pennsylvania Farmers Association, Harrisburg, PA (Mr. M. Eckhaus).

Pennsylvania Farmers Union, Harrisburg, PA (Mr. R. Pennay).

Pennsylvania Federated Humane Societies, Harrisburg, PA (Mr. H.C. Criswell).

Pennsylvania Federation of Sportsmen's Clubs, Inc., Harrisburg, PA (Mr. R. Holman).

Pennsylvania State Department of Agriculture, Harrisburg, PA (Drs. M. Van Buskirk, J. Cable, B. Wolf, N. Buss, G. Schenck).

Pennsylvania State Department of Environmental Resources, Harrisburg, PA (Mr. V.R. McElhattan).

Pennsylvania State Game Commission, Harrisburg, PA (Messrs. D. Sheffer, A. Hayden, B.R. Hambley, B.L. Warner).

Pennsylvania State Grange, Harrisburg, PA (Ms. B. Shambaugh).

Pennsylvania State Livestock Association, Harrisburg, PA (Ms. B. Snyder).

Pennsylvania State Veterinary Medical Association, Harrisburg, PA (Dr. H. Russell).

Philadelphia Department of Health, Philadelphia, PA (Dr. R. Sharrer).

Rhone Merieux, Inc., Lyon, France (Drs. P. Desmettre, G. Chappuis, M. Lombard).

Rhone Merieux, Inc., Athens, GA (Drs. D. Hildebrand, J. Mackie).

Rockefeller University, New York, NY (Dr. S. Morse).

Southeastern Cooperative Wildlife Disease Study, Athens, GA (Drs. V. Nettles, S. Linhart).

Sullivan County Chamber of Commerce, La Porte, PA (Ms. J.R. Mueller).

Sullivan County Medical Center, La Porte, PA (Dr. C.L. Vermeire).

University of Alaska, Institute of Arctic Biology, Fairbanks, AK (Dr. E. Follmann).

University of Georgia, College of Veterinary Medicine, Athens, GA (Dr. D. Dreesen).

University of Liege, Belgium (Drs. P.P. Pastoret, B. Brochier).

University of Pennsylvania, School of Veterinary Medicine, Philadelphia, PA (Dr. A. Hamir).

University of Waterloo, Canada (Dr. S. Smith).

University of Wisconsin-Madison, Department of Veterinary Science, Madison, WI (Drs. T. Yuill, M. Camilo Vargas).

U.S. Animal Health Association, Richmond, VA (Dr. L. Russell).

U.S. Department of Agriculture, Agricultural Biotechnology Research Advisory Committee, Washington, DC (Dr. B. Osborn).

U.S. Department of Agriculture, Agricultural Research Service, Plum Island, NY (Dr. J.J. Callis).

U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, Area Veterinarian-in-Charge, Albany, NY (Dr. G.F. Toms).

U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, Area Veterinarian-in-Charge, Harrisburg, PA (Dr. U.L. Lane).

Utah State University, Department of Fisheries and Wildlife, Logan, UT (Dr. R. Dueser).

Virginia State Department of Health, Richmond, VA (Dr. S. Jenkins).

Washington State University, Pullman, WA (Dr. J. Gorham).

Wildlife Disease Association, Ames, IA (Dr. E. Addison).

Wildlife Refuge Center, Vincentown, NJ (Ms. P. Getson).

World Health Organization, Geneva, Switzerland (Drs. K. Bogel, F. Meslin).

C. APHIS, USDA, in reviewing this application and Environmental Assessment, has consulted the following:

1. National Vaccine Program Office, Vaccinia Subcommittee
   - Dr. Walter E. Brandt, USAMMDA, Chairman
   - Dr. Paul Albrecht, FDA-CBER
   - Dr. Joel Dalrymple, USAMRIID
   - Dr. Elaine Esber, FDA-CBER
   - Dr. William Fitzgerald, FDA-CBER
   - Dr. William Habig, FDA-CBER
   - Dr. Devaraya Jagannata, FDA-CVM
   - Dr. Brian Mahy, CDC-VRD
   - Dr. Caryn Miller, USAID-ST
   - Dr. Bernard Moss, NIH-NIAID
   - Dr. Yuth Nimit, NVP
   - Dr. Hebert Smith, FDA-CBER
2. Ad Hoc Expert Panel
   - Dr. George M. Baer, CDC, Chairman
   - Dr. Marck Buller, NIH
   - Mr. Arnold Hayden, PA Game Comm.
   - Dr. John Hadidian, NPS
   - Dr. Henry Miller, FDA
   - Dr. Robert Wachbroit, Univ. Md

The USDA, Office of Agriculture Biotechnology (OAB) has also received notification regarding this action.

XIII. Preparation of EA

List of preparers of Environmental Assessment within APHIS, Biotechnology, Biologics, and Environmental Protection (BBEP), Mr. T. L. Medley, J.D., Director.

Veterinary Biologics:
- Dr. D. Espeseth
- Dr. R. Miller
- Dr. G. Shibley

Environmental Documentation:
- Mr. M. Werner, J.D.
- Mr. C. Brown
- Mr. C. Divan
- Ms. S. Lowe
- Ms. J. Montgomery
- Ms. P. Rogers
- Mr. B. Sterk
- Dr. O. Young

Biotechnology Coordination and Technical Assistance:
- Mr. M. Lidsky, J.D.
- Dr. F. Tang, J.D.

XIV. Appendices

A. Construction and Characteristics of the V-RG Vaccine Virus

B. Report of Field Trial on Parramore Island, Virginia, 1990

C. Laboratory Evaluation of Non-target Animals

D. Safety Trials with Non-human Primates

E. Immunodeficient Mice Trials

F. Letter from Commonwealth of Pennsylvania

G. Comments on Draft Environmental Assessment

Construction and Characteristics of the V-RG Vaccine Virus

Prepared by:

The Wistar Institute of Anatomy and Biology

Philadelphia, Pennsylvania

For:

The United States Department of Agriculture

February 1, 1991

Construction and Characteristics of the V-RG Vaccine Virus

Double-stranded complementary DNA (cDNA) was synthesized from rabies virus-specific glycoprotein messenger RNA (mRNA) of the Evelyn-Rokitnicki-Abelseth (ERA) strain and cloned into plasmid pBR322 by G/C tailing (1). The "glycoprotein" cDNA contained 1.65 kilobase (kb) pairs and lacked only 21 nucleotides from the 5' terminus of the glycoprotein mRNA. The precise location of the "glycoprotein" cDNA sequence in the glycoprotein gene of rabies virus (ERA strain) was recently determined from the nucleotide sequence of the genome RNA upstream from the 5' end of the glycoprotein mRNA, which includes the intergenic sequence between the matrix protein gene and glycoprotein gene (14). The glycoprotein gene contains 28 non-coding nucleotides at the 5' end of the mRNA and 75 non-coding nucleotides, including the stretch of polyadenylic nucleotides, at the 3' end.

The longest open reading frame of the cDNA nucleotide sequence encodes a polypeptide of 524 amino acids beginning with the initiation codon ATG and ending with the termination codon TGA (1). The first 19 amino acids represent a signal peptide which precedes the sequence lysine-phenylalanine-proline-isoleucine-tyrosine-threonine, which has been identified as the N-terminal sequence of the purified rabies virus glycoprotein (9). In the processing of the polypeptide which is derived endogenously (in the rabies genome RNA) from the glycoprotein gene (or exogenously from cDNA), a signal peptide is cleaved from the N-terminus of the polypeptide, leaving a mature glycoprotein of 505 amino acids. The initial "glycoprotein" cDNA, however, contained the codon for leucine at position 8 of the amino acid sequence of the mature glycoprotein instead of the codon for proline which is the amino acid found in the native mature glycoprotein. This undesirable codon for leucine in the original "glycoprotein" cDNA was corrected and the codon now codes for proline at position 8 (17,18). The mature native glycoprotein derived from the glycoprotein mRNA (or from the "glycoprotein" cDNA as described below) is capable of inducing and binding rabies virus-neutralizing antibodies and also stimulating immune T cells raised by the host immune system.

The upstream G/C tail of the double-stranded cDNA used for cloning the "glycoprotein" cDNA was removed since such tracts may interfere with expression of the encoded protein. A unique MstII restriction site overlapping codons for amino acids 2-4 of the "glycoprotein" cDNA (in the signal peptide sequence) was linked, through a synthetic double-stranded adapter oligonucleotide, to an upstream BglII site located within the plasmid vector of the original cDNA clone.

To construct the V-RG recombinant virus, it was necessary to transfer the "glycoprotein" cDNA to the carrier plasmid which contained nucleotide sequences of the TK gene from vaccinia virus (3,10-13). Specifically, the 4.6-kb HindIII fragment (Hin-J) of the vaccinia virus genome containing the complete TK gene was inserted into the unique HindIII site of an E. coli miniplasmid (ptg1H, 2049 kb) bearing a beta-lactamase gene (conferring ampicillin resistance) and an origin of replication. This construct, ptg1H-TK, was used as the carrier plasmid (8). The carrier plasmid also contains a strong promoter sequence (to drive the expression of the rabies glycoprotein) of the early vaccinia virus gene encoding a 7.5 kilodalton (kDa) protein, linked to the TK gene. The promoter lies on the SolS fragment of the vaccinia virus genome, one of the smallest fragments generated upon digestion of vaccinia virus DNA by SolI. This promoter segment (270 bp) with a BglII linker introduced immediately downstream was inserted into the TK-gene-coding sequence carried by the ptg1H-TK plasmid (8,16). The rabies glycoprotein coding sequence was introduced into a BamHI site immediately downstream of the 7.5 kDa protein gene promoter within the TK gene. By inserting the "glycoprotein" cDNA into the TK gene, the vaccinia virus TK gene is inactivated and the virulence of the recombinant virus is dramatically decreased (5 $\log_{10}$) compared with that of the parent vaccinia virus (2).

The V-RG recombinant virus was constructed by the in vivo recombination exchange of the interrupted inactivated TK gene, containing the "glycoprotein" cDNA insert, in the carrier plasmid ptg1H-TK, for the TK gene of the parental vaccinia virus. A double-reciprocal recombination between the constructed plasmid (bearing the rabies "glycoprotein" cDNA inserted within nucleotide sequences of the vaccinia virus TK gene) and the vaccinia virus genome exchanges the viral TK gene for the insert-bearing TK gene present in the plasmid.

In the Virology Department at the University of Strasbourg, this "Copenhagen" strain of vaccinia virus was cloned on chicken embryo fibroblast (CEF) cells (6,7). The cell culture used for cloning was established from "specific pathogen-free" embryonated eggs from Lohmann (Cuxhaven). From a viral clone, a first virus stock was produced by amplification in CEF cells. A second passage was made on CEF cells and subjected to the mutagen N-methyl N'-nitrosoguanidine according to the protocol of Drillien et al. (6). Temperature-sensitive mutants, tsN7and tsN26 (also referred to as ts26) were isolated and characterized from individual plaques. After a cycle of amplification of the infectious mutants, a second cloning was performed. The virus of this last cloning was passaged 3-4 times on CEF cells to establish working stocks.

Purified vaccinia virus DNA is noninfective, so to generate recombinants it was necessary to introduce the plasmid (DNA) construct into cells simultaneously infected with TK-positive vaccinia viral DNA by calcium-mediated transfection. To further reduce the chance of producing nonrecombinant parental virus in the BUdR selection protocol, the temperature-sensitive mutant ts26 of the original Copenhagen strain was used as the "helper" virus (5). Therefore, when the cells which were competent for plasmid transfection and simultaneously infected with the ts26 mutant were maintained at the nonpermissive temperature (39.5°C) for the ts26 "helper" virus, no yield of "helper" virus was obtained and the detection of the rare TK-negative recombinant virus was considerably enhanced (8,16).

The TK-negative V-RG virus was selected by plating upon a host cell line lacking its own TK gene in the presence of 5-bromodeoxyuridine (5-BUdR). The TK produced by parental virus phosphorylates 5-BUdR to the 5'-monophosphate, which is subsequently converted to the triphosphate. This compound is an analogue of dTTP and its incorporation into DNA prevents the correct development of the parental virus. A TK-negative (recombinant) virus, however, replicates its DNA normally and gives rise to visible plaques on a monolayer of an appropriate TK^- cell line (mouse L-TK).

Analysis of the V-RG recombinant DNA by HindIII restriction enzyme digestion reveals that the 4.6 kb Hin-J fragment (which contains the complete TK gene in the parent vaccinia virus) is absent in the V-RG recombinant virus. Instead, two new fragments,

isolated with HindIII, of approximately 1.1 and 5.5 kb revealed the presence of an insert which bears the unique HindIII site overlapping codons 10 and 11 of the rabies "glycoprotein" cDNA (8,16). The V-RG recombinant virus used in the animal immunization trials bears the technical designation VVTGgRAB-26D3 (16).

The rabies-specific antigen expressed by the V-RG recombinant is shown by SDS-polyacrylamide gel electrophoresis and immunoblotting to be equivalent to authentic rabies glycoprotein from rabies virus. Its apparent molecular mass of about 66 kDa corresponds to the size, 67 kDa, of ERA virus glycoprotein (4). Comparison of the expressed V-RG virus antigen with rabies virus glycoprotein in radioimmunoassay for antigenic reactivity with a panel of 44 anti-rabies glycoprotein Mabs also shows that the two antigens are almost identical (8,16).

The V-RG recombinant virus is propagated in a "Vero" green monkey kidney cell line, established in 1962 by Y. Yasumura and Y. Kawakita in Japan. The cell line is filed with the American Type Cell Collection under CCL 81. A master cell stock has been grown and preserved by freezing in liquid nitrogen. After cloning, a master seed of the V-RG recombinant virus was established. This stock is preserved by freeze-drying. All the vaccine preparations originate from this stock, within the limit of a maximum of 5 passages. The vaccine preparation is a viral suspension which is available either deep-frozen or lyophilized, associated with a stabilizer. Each batch of vaccine is subjected to: a titration of the V-RG recombinant virus by determining the number of$TCID_{50}$ per unit volume; an efficacy test of the V-RG recombinant virus by vaccination of mice, followed 14 days later by a challenge by a virulent rabies virus. This standard method enables a 50% protective dose (PD_{50}) to be calculated; and tests for freedom from adventitious viral and microbial agents.

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Development of an Oral Wildlife Rabies Vaccine:

A Vaccinia-Rabies Glycoprotein (V-RG) Recombinant Virus Field Trial

Parramore Island VA

150-Day Summary Report

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For:

The United States Department of Agriculture

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INTRODUCTION

During the last eight years, The Wistar Institute of Anatomy and Biology (Wistar), Philadelphia, Pennsylvania and its collaborators have been developing a vaccinia-rabies glycoprotein (V-RG) recombinant virus vaccine intended to control rabies in free-ranging terrestrial animals via oral vaccination (for background, see (FR 9241, March 6, 1989). This report summarizes the initial 150 days of the first limited field trial of the V-RG recombinant virus vaccine in North America, that commenced August 20, 1990, on Parramore Island, Virginia.

Two days prior to initiation of the field trial, the V-RG recombinant vaccine was prepared in the laboratories of Wistar's Rabies Unit. Lyophilized vaccine (Lot # 5L24) was stored at 4°C before reconstitution in sterile distilled water. Vaccine was prepared for a final approximate dilution of 10^8 pfu/mL in sterile PBS and 1.0 mL was instilled into paraffin ampules. Ampules were sealed with wax and each was inserted into a fishmeal polymer bait cylinder containing 150 mg of tetracycline in its matrix, which was then plugged and sealed with wax. Baits were placed into individually numbered polyethylene bags, bearing a descriptive label approved by the United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service, as to the contents. Bagged baits were sealed into 18-liter drums carrying a biohazard identification label. The following day, Wistar personnel took the refrigerated drums to the test area by vehicle. They were then transported to the island by boat.

On August 20, 1990, a total of 3,120 vaccine-laden baits were distributed by hand over approximately 312 ha of Parramore Island. In addition, 50 mL of a slurry (consisting of H_2O, sucrose, domestic fowl egg yolks, vegetable oil, homogenized blue crab, and 250 mg of sulfadimethoxine) were added to each bag to enhance the bait's attractiveness to raccoons. Baits were placed at pre-determined, individually-numbered, flagged locations in upland forest, thicket-marsh interface and thicket-dune interface, in as close to a linear pattern as possible, given local topography, and concentrated on areas having signs suggestive of raccoon activity such as tracks, scat, dens, or trails.

BAIT DISTURBANCE

One objective of the field trial was to observe and record the rate of vaccine-laden bait disturbance and to approximate the proportion of baits contacted by the target species, as well as by other animals such as foxes, small mammals and avian species. In order to document disturbance of the fishmeal polymer baits, bags, and vaccine-laden ampules, bait stations were individually checked during the vaccine trial. Individual records were kept on

each of the 3,120 bait stations. Records from the bait stations revealed, that within 48 hours of distribution, approximately 50% of the baits had evidence of animal contact. This ranged from complete disappearance of the bait and bag from the bait station to finding only a torn or chewed bag, remnants of the fishmeal polymer bait or chewed or punctured remains of the wax ampule. Based upon the fate of the wax ampule containing vaccine, more than 90% of the vaccine-laden baits had been disturbed by day 5 (Figure 1). Studies conducted prior to vaccine application included data that identified raccoons as the most abundant carnivore on the island. With an estimated density of one raccoon per 2.7 ha, the probability of these animals contacting a majority of the baits was high. During the field trial, the majority of identifiable tooth or chew marks on bait remains were compatible with marks made by carnivores. These results further supported a conclusion that raccoons contacted most of the baits.

BAIT CONTACT

To augment identification of animal species contacting baits, tracking pits were established at randomly chosen bait stations in each of the six sections of the vaccination area. The pits were checked daily for bait disturbance. In addition, photographic and written records were taken on discernable animal tracks that could be characterized as carnivore, small mammal, ungulate or avian, in origin. On Parramore Island, naturally occurring carnivores include only raccoons (Procyon lotor) and red foxes (Vulpes vulpes); the only ungulates are white-tailed deer (Odocoileus virginianus). Four small mammal species are present: the rice rat (Oryzomys palustris), house mouse (Mus musculus), meadow vole (Microtus pennsylvanicus) and Norway rat (Rattus norvegicus). Based on tracking pit data obtained from 100 stations in the vaccination area, carnivores were the major species implicated in the disturbance and consumption of vaccine-laden fishmeal polymer baits. Carnivore tracks were further characterized as either raccoon or fox; the majority (>75%) were raccoon (Figure 2). For example, in one section of the vaccination area, all the baits from 20 tracking pits were completely gone within 24 hours; 18 of these pits contained raccoon tracks. Additionally, no vultures, fish crows, gulls, or other avian species were directly observed in the vaccination area in the specific vicinity of the baits.

ANIMAL CAPTURES

Raccoons

Another objective of the field trial was to assess any adverse clinical signs or pathology attributable to the vaccine in free-ranging animals in the vaccination area compared to animals in the surveillance areas, where no baits were distributed. Animals

were collected for assessment primarily through live trapping or by shooting, when appropriate. During the first 30 days of the field trial, the cumulative number of raccoons captured in the vaccination area rose consistently at a mean rate of 10.5 raccoons for each night that traps were set. Tomahawk live traps were set in the vaccination area on 20 of the first 30 days of the field trial, at an average of approximately 150 per night and up to a maximum of approximately 215 per night. In the vaccination area, a total of 209 raccoons (104 individuals plus 105 recaptures) were live trapped and evaluated from August 20 through September 20, 1990. In the surveillance areas, which included the control areas where no baits were distributed, the cumulative number of raccoon captures also rose consistently, but at a lower rate, compared to the vaccination area, of 2.3 raccoons per night that traps were set, due to the proportionately smaller areas under surveillance. In the surveillance areas, 39 raccoons (26 individuals plus 13 recaptures) were live trapped and evaluated during the first 30 days. The cumulative numbers of captured raccoons continued to rise in both the vaccination and surveillance areas, clearly demonstrating that raccoons were present in both baited and control areas and were not experiencing illness or mortality that would adversely affect trap success.

All live-trapped raccoons were routinely sedated and thoroughly examined. The examination included specific evaluation for theoretical, but as yet unrealized, manifestations of adverse effects of the vaccine in raccoons, such as an abnormal attitude mentation, depression, dullness, gross lesions including ocular, or other mucous membranes, or weight loss. Samples taken during the exam included blood for serology and oral and fecal swabs taken for virus isolation during the first two weeks of the trial. All live-trapped raccoons were found to be within normal limits with respect to attitude and appearence upon physical examination. Additionally, there was no significant difference between the mean weight of live-trapped raccoons grouped according to age class in the vaccination area and those live trapped in the surveillance area during the first 30 days (student's test, $p>0.05$).

During the period August 20 through October 31, 1990, 416 live-captured raccoons were examined island-wide. In the surveillance areas, live trapping resulted in the initial capture of 55 raccoons plus 30 recaptures. Since the beginning of the field trial, 134 raccoons were live trapped in the vaccination area in addition to 197 recaptures (Table 1). None of these animals was remarkable upon physical examination. The "resident" population or "population at risk," defined as those animals live trapped in the vaccination area during the first two weeks of the field trial, consisted of 100 raccoons. Additionally, 34 "adjunct resident" raccoons were captured in the vaccination area after September 3. By October 31, 1990, over 65 residents had been recaptured, re-bled and re-examined at least

once since the initiation of the field trial. Upon recapture, no physical abnormalities were noted.

Between August 20 and November 30, 1990, 492 captures occurred in the vaccination and surveillance areas (Table 1). In the first three calender months of the Parramore Island field trial, this consisted of 205 live-captured individuals and 287 recaptured raccoons, with an overall effort of 3,521 trap-nights (14.0% trap success). In the vaccination area, 141 individuals and 238 recaptured raccoons were live trapped, with an overall effort of 2,239 trap-nights (16.9% trap success). This compares to a capture of 113 raccoons, composed of 64 individuals and 49 recaptures, with an effort of 1,282 trap-nights (8.8% overall trap success) in the four surveillance areas. None of these animals was remarkable upon physical examination.

From August 20 to December 31, 1990, 530 total captures occurred in the vaccination and surveillance areas (Table 1). In the first four calender months of the Parramore Island field trial, this consisted of 221 individuals and 309 recaptured raccoons, with an overall effort of 997 trap-nights (11.7% trap success). In the vaccination area, 146 individuals and 242 recaptured raccoons were live trapped, with an overall effort of 2,853 trap-nights (13.6% trap success). This compares to a capture of 142 raccoons, composed of 75 individuals and 67 recaptures, with an effort of 1,665 trap-nights (8.5% overall trap success) in the four surveillance areas. None of these animals was remarkable upon physical examination for pox viral-suggestive lesions.

Between the trial's initiation on August 20, 1990, and January 31, 1991, 560 total captures occurred in the vaccination and surveillance areas (Table 1). In the first five calendar months of the Parramore Island field trial, this consisted of 234 individuals and 326 recaptured raccoons, with an overall effort of 4,968 trap-nights (11.3% trap success). In the vaccination area, 151 individual raccoons and 248 recaptured raccoons were live trapped with an effort of 3,129 trap-nights (12.7% overall trap success). This compares to a capture of 161 raccoons, composed of 83 animals and 78 recaptures, with an effort of 1,839 trap-nights (8.8% overall trap success) in the four surveillance areas. None of these animals was remarkable at physical examination during routine observation for pox viral-suggestive lesions.

Small Mammals

Sherman box traps are being used to live trap non-target rodent species to monitor them for potential adverse effects from the V-RG virus vaccine. During the first two weeks of the trial, 36 rodents were live trapped in the vaccination area and 11 were captured from surveillance areas. All animals were clinically normal, with neither evidence of adverse

behavioral signs nor gross lesions suggestive of vaccine-induced morbidity. Small mammal trapping efforts shifted from lines in August and September to primarily a grid method in October in order to maximize individual recaptures over time. During October, a total of 2,049 trap-nights for small mammals along six linear areas and on 15 grids (nine in the vaccination area, six in surveillance areas) resulted in the capture of 84 rodents, eight being recaptures (Table 2). These rodents included 64 rice rats, 11 meadow voles , 7 house mice and a single Norway rat. Sherman trap disturbance, primarily by raccoons and to a lesser extent by ghost crabs, was substantial, especially in grids located in section one of the vaccination area. No gross lesions suggestive of any viral-induced pathology were observed in physical examination of live-trapped rodents.

During November 1990, 300 small mammals, including 152 new animals and 148 recaptured rodents, were live trapped in the vaccination and surveillance areas (Table 2). The capture effort consisted of 2,841 trap-nights (10.6% trap success). The small mammal species consisted of 242 rice rats, 47 house mice, 6 meadow voles, and 5 Norway rats. In the vaccination area, 199 small mammals were captured, including 182 rice rats (72 new , 110 recaptures), 8 house mice (6 new, 2 recaptures), 4 meadow voles and 5 Norway rats. The capture effort in the vaccination area was 1,891 trap-nights (10.5% trap success) over 12 individual grids. In the surveillance areas, 101 total rodents were live trapped, including 60 rice rats (32 new , 28 recaptures), 39 house mice (31 new, 8 recaptures) and 2 meadow voles. The capture effort for the surveillance areas was 850 trap-nights (11.9% trap success) over 8 individual grids. No trapped rodents presented abnormal clinical behavior or gross lesions consistent with pox viral-related pathology.

During December 1990, 263 small mammals of four species were live trapped in the vaccination and surveillance areas, including 86 new animals and 177 recaptured rodents (Table 2). The capture effort consisted of 3,003 trap-nights (8.8% trap success). The small mammal species consisted of 175 rice rats, 84 house mice, 1 meadow vole and 3 Norway rats. In the vaccination area, 137 small mammals were captured, including 119 rice rats (21 new, 98 recaptures), 15 house mice (7 new, 8 recaptures), 1 meadow vole and 2 Norway rats. The capture effort in the vaccination area was 2,103 trap-nights (6.5% trap success) over 12 individual grids. In the surveillance areas, 126 total rodents were live trapped, consisting of 56 rice rats (26 new, 30 recaptures), 69 house mice (28 new , 41 recaptures) and a Norway rat. The capture effort for the surveillance areas was 900 trap-nights (14.0% trap success) over 8 individual grids. No trapped rodents presented abberant clinical behavior or gross lesions consistent with pox viral-related pathology.

During January 1991, 69 small mammals of three species were live trapped in the vaccination and surveillance areas, including 21 new animals and 48 recaptured rodents

(Table 2). The capture effort consisted of 1,250 trap-nights (5.5% trap success). The small mammal species consisted of 50 rice rats, 18 house mice and 1 meadow vole. In the vaccination area, 47 small mammals were captured, including41 rice rats (10 new , 31 recaptures), 5 house mice (3 new, 2 recaptures) and one meadow vole. The capture effort in the vaccination area was 800 trap-nights (5.9% trap success) over 12 individual grids. In the surveillance areas, 22 total rodents were live trapped, consisting of 9 rice rats (9 recaptures) and 13 house mice (7 new, 6 recaptures). The capture effort for the surveillance areas was 450 trap nights (4.9% trap success) over 8 individual grids. No trapped rodents presented unusual clinical behavior or gross lesions consistent with pox viral-related pathology.

Other Non-target Species

Non-target species surveys focus primarily on small mammals because they are the most abundant non-target animals on site. However, other non-target species on the island include white-tailed deer and red fox. Three red foxes were live trapped, sedated, ear-tagged, bled, radio-collared, physically evaluated, and released during the first two weeks of the trial. Additionally, three white-tailed deer and one red fox were collected for tetracycline analysis and gross and histopathological examination, as well as for virus isolation attempts. These animals were collected from the vaccination area and the south surveillance area, respectively. All bone samples were negative for tetracycline and sera samples were negative for the sulfadimethoxine biomarker. Oral and fecal swabs from the red fox were negative upon virus isolation; the white-tailed deer swab results are pending. No gross lesions were noted during necropsy. Results of the biomarker and histopathological examinations are in Appendix I.

BIOMARKERS

Tetracycline

The calciphillic biomarker tetracycline was included as a long-term post-mortem indicator of bait consumption by an animal. Bone samples are routinely collected from euthanized animals and from carcasses found in the field. In the first 30 days, 12 raccoons, 11 house mice, 4 rice rats, 2 meadow voles and 3 white-tailed deer were euthanized from the vaccination area as compared to 4 raccoons, 1 fox and 5 rodents as negative controls. All animals were healthy with no gross evidence suggestive of orthopoxvirus lesions at the time of necropsy. None of 4 raccoons from surveillance areas were found to be tetracycline positive, compared with 11 of 12 (92%) raccoons from the vaccination area, indicating high bait consumption by raccoons. All of the deer and fox

samples from the first two weeks of the trial were tetracycline negative. All of the rice rat (13) and meadow vole (5) samples collected during this period were also negative for tetracyline. As of January 31, 1991, 2 of 70 (2.8%) of the small mammals examined (14 from surveillance areas, 56 from the vaccination area), a single Rattus and a Mus, were tetracycline positive, indicating apparent bait contact. Blood samples were unavailable from these two animals, as both were first-time capture trap mortalities from the vaccination area. Both rodent carcasses were unremarkable at gross necropsy. As of January 31, 1991, 22 of 25 (88%) of raccoons collected from the vaccination area were tetracycline positive. Conversely, all 14 raccoons from the surveillance areas were tetracycline-negative.

Additionally, other non-target species were evaluated for evidence of tetracycline ingestion. Bone samples were collected from four deer carcasses found in the vaccination area and one fox carcass found in the surveillance area in November and December 1990. All of these bone samples were tetracycline negative.

Sulfadimethoxine

The antibiotic sulfadimethoxine (SDM) was included as short-term ante-mortem serum marker in the slurry as a second indicator of bait contact. Sulfadimethoxine is a short-term serum marker; laboratory studies indicated that blood levels in raccoons given 100 mg and 250 mg of SDM dropped to nearly undetectable levels, using a commercial card test, by day 7. Thus, the relative SDM contact ratio was determined from sera collected from first-time captured raccoons during the first six days of the trial. Sulfadimethoxine analysis was conducted on all sera from captured and recaptured animals to follow decay kinetics of the biomarker in the field. The bait contact ratio was estimated to be 77.5% in 38 of 49 sera, based on cumulative SDM results from first-time captured raccoons during the first six days of the field trial. The frequency of positives was slightly higher (79%, 42 of 53 sera) when samples from recaptured animals already known to be SDM-positive were included in the cumulative six-day rate. Thereafter, as blood-marker levels declined over the following week, only four serum samples of over 100 raccoons examined from the vaccination area were found to be positive for SDM. In contrast to the high frequency of positives in the vaccination area, all sera collected from raccoons in the surveillance areas during the first six days of the field trial were negative (0 of 12 sera) for SDM. During the first two weeks of the trial, none of 32 raccoon nor 4 red fox sera collected from surveillance areas were positive for SDM.

SEROLOGY

Analysis of individual raccoon sera from the vaccination area on days 13 and 14 of the trial demonstrated that 4 of 17 (24%) animals already had significant levels of VNA, which were in excess of 6.0 IU/mL. Two of these animals were also positive for the SDM biomarker when they were originally captured during the first week of the trial (indicating bait contact), but had become negative for the serum marker by day 14. By the third week of the trial, 8 of 15 (53%) individual raccoon sera examined from the vaccination area had evidence of rabies-specific VNA. Six of these eight vaccinates had been previously captured during the first week of the trial; of these six, four had been SDM positive. The sero-prevalence of rabies VNA among recaptured "resident" and "adjunct resident" raccoons handled during the first 60 days was approximately 58.3% (35 of 60), reflecting apparent contact with the vaccine and sero-conversion. Multiple recaptures of 15 individual raccoons allowed for serial bleeds and determination of rabies VNA between days 8 and 63. Geometric mean titers of rabies VNA, based upon these serial bleeds, were highest during the first two weeks at 18.6 IU/mL and began to decline after 30 days to 10.7 IU/mL; to 4.7 IU/mL by 6 weeks; and to 1.4 IU/mL by 60 days.

As of January 31, 1991, sero-prevalence of rabies VNA among resident raccoons from the vaccination area (sections one through six) was approximately 52% (24 of 46), reflecting apparent V-RG vaccine-laden bait contact and sero-conversion (Table 3). All live-trapped raccoons tested to date from surveillance areas (Little Beach, north and south surveillance sections of Parramore Island and Revel's Island) were rabies-antibody negative (N=34), as were all examined to date (N=14) for the biomarker tetracycline.

Although no known vaccine-induced mortality or morbidity has been observed, if the V-RG vaccine had a less obvious effect upon various age and sex classes or upon individual weight gain, differences in life history distribution or animal mass might be expected in sero-positive versus sero-negative animals. However, comparing the relative age and sex categorization of all antibody-positive raccoons (N=39, including adjunct residents) to those antibody-negative raccoons captured from both the vaccination and surveillance areas (N=60), there was no statistically significant difference ($p < 0.05$) in the Chi-square distribution by age and sex of sero-positive versus sero-negative animals. Additionally, there was no significant difference ($p < 0.05$) in the mean ($\pm$ s.d) weight comparison of antibody-positive raccoons (3.2 kg $\pm$ 1.2, N = 39) versus antibody-negative raccoons (3.7 kg $\pm$ 1.6, N = 60) from the vaccination and surveillance areas.

To date, 221 small mammal sera (155 rice rats, 41 house mice, 23 meadow voles and 2 Norway rats), have been tested for rabies VNA. No rabies VNA activity ($\geq$ complete

neutralization at a 1/5 serum dilution) has been detected in any of these small mammal samples (158 from the vaccination area, 63 from the surveillance areas).

RADIOTELEMETRY

At the end of the first month, 40 functional radio-collars with activity and mortality functions remained in place on raccoons captured before and during the first 14 days of the field trial. The majority (38 of 40) of raccoons with radio-collars were located daily. The remaining two animals were located weekly, but only with considerable effort, perhaps due to behavioral variables of the animals such as different denning characteristics or variation of transmitter range in relation to topography. No mortality or morbidity was observed among any of the radio-collared raccoons.

Radiotelemetry monitoring, with transmitters containing activity and mortality functions, continued at least weekly; 40 radio-collared animals were monitored through September 20, 1990 (Table 4). From mid-September to October 31, six raccoons and one fox had removed or lost their collars, as indicated by mortality signals and subsequent location and examination of the collars. Of the remaining 33 radio-collared animals, 4 red foxes and 27 raccoons had been routinely located mid-September through October 31, 1990, with two exceptions. Two raccoons from the vaccination area were found with their radio-collars in mortality mode during the last two weeks of October. One raccoon (eartag # 8217-18) was last handled on October 12 and was found to be in mortality two weeks later. When it was located on October 30, it was unsuitable for gross necropsy, histopathological evaluation, or virus isolation because the carcass was more than 48 hours old. Although the carcass was too old to determine the cause of death, given the posture of the animal and the proximity of the carcass (within 100 meters) to its most recent trap site, and because it had been bled via anterior vena cava or cardiac puncture rather than by jugular venipuncture when it was handled on October 12, it is probable that the animal died from blood sampling rather than natural causes. There was suitable hard tissue (mandible and femur) for tetracycline analysis; the raccoon was tetracycline positive and positive for rabies VNA. This raccoon was part of the "resident" population in the vaccination area. The second raccoon (eartag # 6784-85), with a radio-collar that went into mortality mode during October, had been collared prior to the vaccine field trial (June 14, 1990). The last successful radio locations confirmed the animal alive on October 10 and 11. Again, the carcass was more than 48 hours old when located on October 19 and had also been disturbed by other animals, presumably red fox; thus, it was unsuitable for gross necropsy, histopathological evaluation, or virus isolation. Hard tissue (mandible and femur) was collected and was found to be negative for tetracycline. This raccoon was not considered

part of the resident population or the "population at risk" for eating a bait during the vaccine trial because it was not live trapped in the vaccination area during the first two weeks of the trial.

Through November, 25 functional radio-collars remained on raccoons in the vaccination and surveillance areas (Table 4). One of these collars was recovered by researchers in the field after apparent slippage or removal by the animal. Another transmitter was purposely removed by research personnel during live trapping, due to normal field wear of the collar and the need for refurbishment. Of the remaining 23 radio-collars, 22 were successfully located on a regular basis. No signal was received from the remaining transmitter, although attempts continued to actively recover the animal. Of the original five radio-collars on red foxes at the initiation of the field trial, only four were intact by the end of September, one having slipped from the animal's neck. Of these latter four foxes, all had been actively located by the end of November.

Through December, 23 functional radio-collars remained on raccoons in the vaccination and surveillance areas and one additional raccoon was radio collared (Table 4). One of these collars was recovered by researchers in the field after apparent slippage or removal by the animal. Of the remaining 23 radio-collars, 22 were successfully located on a regular basis. No signal was received from the remaining transmitter, although attempts continued to actively recover the animal. Of the four red foxes collared at the beginning of the month, all were actively located by the end of December.

At the begining of January, 23 functional radio-collars remained on raccoons in the vaccination and surveillance areas (Table 4). One of the collars was noted to be in mortality mode on January 8, 1991. The next day, the body of the radio-collared raccoon (ear tag # 8006-8308) was recovered and noted to be emaciated. The animal was in apparent good health when last handled on November 30. Bone samples taken for tetracycline analysis were positive and a previous serum sample was positive for rabies VNA. The rest of the carcass was placed in fixative for possible histopathological examination.

Of the remaining 22 radio-collared animals, 20 were successfully located and recorded to be in activity mode. Transmissions from two radio-collared raccoons were not received during January 1991. Radio-collar frequency 151.764 represents an adult female raccoon collared in the vaccination area during the first two weeks of the field trial; it was located last on October 18, 1990. Current disposition of this raccoon is unknown. Its radio-collar frequency is still routinely searched but it is possible that this animal may not be relocated. The remaining transmitter that was not located in January represents an adult male raccoon (ear tag# 8020-8021) from section four of the vaccination area. In the past, this raccoon has occasionally been difficult to locate, perhaps due to physical characteristics

of either denning or foraging sites that may be affecting transmission from the radio-collar. This animal's radio-collar frequency is still being actively monitored. All four of the red foxes collared at the beginning of the trial have been routinely located through the end of January.

CARCASS RECOVERY

Despite at least a dozen researchers conducting active surveillance, spending 10 to 12 hours per day on site during days 0 to 14 of the trial, and on days 15 to 30, several researchers spending approximately 8 to 12 hours per day on site, no fresh carcasses of dead animals were discovered in either the vaccination or surveillance areas during the first month.

In October, two radio-collared raccoons (ear tag # 6784-6785 and 8217-8218) were found dead in the vaccination area. Raccoon number 6784-6785 had been captured and collared prior to August 20, 1990. It was found dead on the beach in section one of the vaccination area. The carcass was unsuitable for gross or histopathological analysis. Bone samples from this raccoon were tetracycline negative. Raccoon number 8217-8218 had been recently examined and bled. It was tetracycline and antibody positive. A traumatic bleeding technique was implicated in its death; tissues were unsuitable for further examination.

During routine field surveillance on November 17, two raccoon carcasses were found washed up in debris on the beach in section one of the vaccination area following an unusually high tide. One raccoon was a juvenile male (ear tag #8120-8121) and the second was an adult female (ear tag # 8289-8290). Both had been initially captured, and 8120-21 had been recaptured, during the first 14 days of the field trial. Although the raccoons appeared to have died from hypothermia or drowning, the carcasses were unsuitable for gross or histopathological examination. However, bone samples were collected for biomarker analysis; both were tetracycline positive. The antibody status of both raccoons remains undetermined because they had not been recaptured and re-bled, after the first two weeks of the trial.

On December 11, a raccoon (ear tag # 6880-6881) was found in the marsh in section five of the vaccination area following an unusually high tide. A serum sample collected on October 11 was negative for rabies VNA (0.07 IU/mL). Bone samples from this raccoon were tetracycline positive. The carcass was unsuitable for gross or histopathologic evaluation.

During routine live trapping on December 20, an emaciated raccoon was found dead in a trap in the Revel's Island surveillance area. This raccoon was a subadult female (ear

tag # 8167-8168). The carcass was placed in fixative for possible histopathological examination. However, bone samples were collected for biomarker analysis and were tetracycline negative. The antibody status of the raccoon remains undetermined because no serum was obtainable from the carcass and the raccoon had not been previously captured.

During routine field surveillance in January 1991, a non-eartagged, decomposed raccoon carcass (ID# 8499) was found in the north surveillance area (section seven). Bone samples were collected for biomarker analysis and were tetracycline-negative. Also, a radio-collared raccoon (ear tag # 8006 - 8308) was found dead in the vaccination area. The carcass was emaciated. The raccoon was tetracyclin-positive and antibody-positive. Cumulative results of carcass recovery demonstrate no apparent trend of raccoon mortality associated with biomarker status, and hence at risk of vaccine exposure.

GROSS NECROPSY AND HISTOPATHOLOGY

Eighteen live-trapped raccoons and one trap mortality from the vaccination area and 13 live-trapped raccoons from the surveillance areas were collected for euthanasia, gross and histopathological examination, virus isolation, and tetracycline analysis (Appendix 1). Live trapped raccoons were euthanized by an intravenous administration of a concentrated sodium barbiturate solution. All live-trapped animals were non-remarkable, with no evidence of unusual mentation, localized macular-papular rash, prostration, pneumonia, or toxemia, upon initial physical examination. Upon post-mortem examination, no gross lesions were detected that were compatible with an infectious viral etiology. Blood was collected for a determination of rabies VNA titer, as previously described. Two of the 12 raccoons from the vaccination area that were euthanized during the first two weeks of the trial had already developed rabies VNA titers. Portions of brainstem, lung, liver, spleen, kidney and tonsil were obtained from individual animals for V-RG vaccine virus isolation attempts. Additionally, brain and other tissue samples of representative major organs were fixed in 10% (vol/vol) buffered formalin, embedded in paraffin, sectioned at 6 μm and stained with hematoxylin and eosin prior to examination by light microscopy. Routine tissue examination included a minimum of 37 tissue sections per animal except as noted, including: heart (right and left ventricle); diaphragm; tongue; masseter muscle; lung; liver; gall bladder; kidney; pancreas; spleen; skin (body and paw); thymus; eye; mesenteric lymph node (3 sections); stomach; intestines (minimum 4 sections); salivary glands; urinary bladder; prostate; testicle; ovary; uterus; trachea; thyroid; esophagus (2 sections); aorta; brain (3 sections); and cervical spinal cord.

Incidental skin lesions were found in 62.5% of raccoons (5 of 8) from the surveillance areas and in 66.7% of raccoons (12 of 18) from the vaccination area.

Raccoons from the surveillance area had lesions such as: mild, focal to multifocal dermatitis or epidermitis with bacteria present (8001-02, 8659-60, 8126-27); one focal area of skin ulceration and subjacent cellulitis on the leg (6823-24); and an old traumatic lesion on the bridge of the nose that was ulcerated and fibrotic (6870-71). Raccoons from the vaccination area had lesions consistent with various etiologies, as listed below:

- Ticks present; focal area dermatitis with mild focal sero-cellular crust and mild infiltrate in the subjacent dermis (8505-06);
- no ticks present but consistent with mild, multifocal mononuclear infiltrate in the superficial dermis and a small focal sero-cellular crust over the epidermis on the body. The skin of the paw had multifocal areas of hyperplasia present (8611-12);
- Demodex present; no cellular reaction (8009-10, 8337-38);
- unidentified parasite; one focal area dermal infiltrate (8220-21);
- trauma; multifocal chronic ulcerative dermatitis on the skin of the face with plant material within the reaction (8513-14). Skin around lower leg wound severe, chronic, locally extensive cellulitis with many bacterial colonies (8625-26);
- bacteria present; multifocal dermal thrombosis and vasculitis with bacterial colonies consistent with partial autolysis of tissues due to death 12-36 hours prior to post-mortem.
- mild, focal perifolliculitis (8655-56) present on the paw; small focal areas of mild dermatitis (8255-56); mild increase in the number of cells in the dermis but normal otherwise (6851-8025);
- chronic, active dermatitis or cellulitis on the head (6862-63).

In summary, no gross or histopathological lesions referable to the V-RG recombinant virus vaccine have been observed to date (Appendix 1).

VIRUS ISOLATION

Tissues

Virus isolation has been completed on 11 sets of tissues (blood, tonsil, lung, liver, spleen, kidney, and brain) from eight live-trapped raccoons in the vaccination area and three raccoons from surveillance areas (Appendix I). Virus was recovered from only the tonsils of two raccoons (ear tag # 6884-6885 and ear tag # 8513-14, both biomarker positive) trapped in the vaccination area and euthanized on the second and fourth day of the vaccine trial, respectively. The necropsies from these animals were non-remarkable. Virus isolation attempts on tissues from the remaining animals are in progress.

Oral and Fecal Swabs

To date, 71 swabs (48 oral, 23 fecal) have been tested in cell culture for virus isolation collected from 47 raccoons and a red fox during the first six days of the vaccine trial. These consisted of 10 individuals from the surveillance areas and 37 individual raccoons from the vaccination area, the latter including 30 raccoons that were biomarker (SDM and/or tetracycline) positive. Results of all oral and fecal swabs tested were negative for virus isolation, regardless of animal origin or biomarker or serological status.

ENVIRONMENTAL STABILITY OF VACCINE

In order to monitor the rate of vaccine virus deterioration under various environmental conditions on Parramore Island, screened enclosures were constructed to protect baits from direct animal molestation. These enclosures were placed in closed Tomahawk traps located in the vaccination area under conditions of full sun to full shade. Fourteen vaccine-laden baits were placed in each individual enclosure and air temperature variations were monitored by means of a maximum-minimum thermometer. One bait was removed each day and was frozen in a container of dry ice and air temperatures at the time of bait removal were recorded. Frozen baits were transported to the laboratory at Wistar for determination of V-RG recombinant virus titers. Baits were sectioned and the frozen pellets of virus were thawed rapidly and filtered. Serial dilutions were prepared for vaccine titer comparison to vaccine frozen on the original day of laboratory preparation. Frozen aliquots were also sent to the United States Department of Agriculture, National Veterinary Services Laboratory in Ames, Iowa, for comparative evaluation.

Air temperatures during the first 14 days ranged from 19° to 33°C, with direct solar radiation capable of raising thermometer temperatures to 44°C at stability stations in the inter-dune clearings near the sand surface. Precipitation in the form of rain occurred during most of the first week. This combination of alternating wet-dry, cool-hot cycles on Parramore Island softened the bait matrices and allowed fungal growth directly on some of the baits after one week. In areas where the stability station was located in the open without vegetative cover such as on dunes, carrion beetles disrupted the protective screening and were observed consuming the fishmeal polymer baits, together with flies, which deposited eggs on the bait. As much as 25% of a given bait was missing from the screened enclosures by day 14 due to combined effect of weather and invertebrate action. Such arthropod activity apparently did not disturb the interior wax ampule containing the vaccine. Despite these often harsh local environmental conditions, the loss of V-RG virus titer was minimal in the intact wax ampules within these baits, revealing the thermostability

of the vaccine. The V-RG virus had an initial titer of $10^{8.6}$ TCID that decreased to $10^{8.3}$ TCID in partial sunlight and $10^{7.7}$ TCID in full sunlight in a 15-day period; titer loss in two weeks from baits in fully shaded conditions was negligible.

HUMAN SAFETY

During the first two weeks of the trial, as many as 25 researchers took part in the vaccination project. These included visiting American, German, Chinese, Swiss and Canadian scientists, with multi-disciplinary biomedical backgrounds representing the Animal Diseases Research Institute (Ottawa, Canada); the Ontario Ministry of Natural Resources (Toronto, Canada); the Southeastern Cooperative Wildlife Disease Study (Athens, Georgia); the University of Berne (Berne, Switzerland); the University of Virginia (Charlottesville,Virginia); and The Wistar Institute. Although these individuals were at the greatest potential risk of V-RG virus exposure due to contact with thousands of vaccine doses, vaccine-laden baits, and animals presumably in contact with vaccine, all researchers have remained healthy with no suspected vaccine-induced morbidity or lesions. Serum collected from a sample of these individuals pre- and post-trial were screened for the development of any putative anamnestic reactions from such exposure. No serum antibody rise has been detected from paired sera examined to date (N=14).

In the first week of the trial, one researcher was bitten on the index finger by a raccoon that had consumed a vaccine-laden bait within the previous 12 hours. This occurred in the vaccination area, during a live-trapped raccoon's revival from parenteral sedation. The local wound area of the researcher was immediately cleansed and the patient received a thorough physical examination from the local physician on call for the vaccine trial; the wound has since healed uneventfully. No vaccinia-associated lesion has developed. Blood was obtained for serology and no serum antibody rise has been detected in this researcher.

Coast Guard personnel and local Virginia game department wardens on Parramore Island during the trial, were bled before the experiment began and after all baits had been consumed or removed, for example, a minimum of 30 days later. These sera were compared for evidence of inadvertent exposure to the V-RG recombinant virus vaccine. No VNA have been detected. These personnel are not considered to be at direct risk for exposure and are being currently monitored for indirect exposure. This population serves as a control sample for individuals who are physically near the vaccination site yet are not in direct contact with either baits or vaccinated animals.

While vaccine-laden baits were accessible to animals in the field for the first 10 days, dawn to dusk patrols via boat were provided by wardens of the Virginia game

department. This was supplemented by researchers in radio contact in the vaccination and surveillance areas, as well as by foot patrols at potential docking sites on the island. In such areas, prominent signs described the nature of the trial and the closure of the island, which is normally closed to the general public. No trespassing incidents were observed during the field trial, which has proceeded with no untoward events related either to animal safety or site biosecurity.

CONCLUSIONS

Thus far, there have been no identified or suspected mortalities, morbidities, aberrant clinical signs, unusual activities, detrimental effects upon weight gain or age class distributions, gross lesions or histopathological lesions directly attributable to the V-RG recombinant virus vaccine in any target or non-target mammalian species observed on Parramore Island. Despite pronounced bait disturbance (Figure 1), largely attributable to raccoons based upon tracking pit data (Figure 2), and high biomarker (~80%) and sero-conversion rates (50 to 65%, Table 3) among raccoons, suggesting ingestion of multiple vaccine doses by individual raccoons, no negative viral-induced effects have been recorded in the Parramore Island ecosystem to date.

APPENDIX I - 150-DAY REPORT

V-RG VACCINE FIELD TRIAL ON PARRAMORE ISLAND, VA

HISTOPATHOLOGIC FINDINGS, BIOMARKER, VIRUS ISOLATION AND RABIES ANTIBODY STATUS OF EUTHANIZED RACCOONS

1. 90-R36 (8301-02). Female, adult, 8/21/90:
   Surveillance area (SDM and tetracycline negative, antibody negative as of day 1).
   Virus isolation: All tissues negative.

-Heart, tongue and M. muscle - all with a few granulomas (Hepatozoon).
-(Diaphragm and tonsil - not collected for histopathology).

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2. 90-R34 (8001-02). Male, sub-adult, 8/22/90:
   Surveillance area (SDM and tetracycline negative, antibody negative as of day 2).
   Virus isolation: All tissues negative.

-Heart, diaphragm, tongue and M. muscle - all with a few granulomas (Hepatozoon).
-Pancreas - one fluke in duct (Eurytrema).
-Skin (body) - one small area of ulceration and some cellular infiltrate.
-Skin (paw) - one small focal area of dermatitis with a few bacterial colonies.
-Intestines - a few flukes (Phagicolla).
-(Tonsil - not collected for histopathology).

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3. 90-R24 (6851-8025). Male, adult, 8/22/90:
   Vaccination area (SDM postive and tetracycline positive, antibody negative as of day 2).
   Virus isolation: All tissues negative.

-Heart, tongue and M. muscle - all with a few granulomas (Hepatozoon).
-Tongue and M. muscle - a few Sarcocysts.
-Tongue - a few Capillaria.
-Intestines - moderate numbers of flukes (Phagicolla).
-Skin (body) - mild increase in the number of cells in the dermis, but normal otherwise.
-(Diaphragm and salivary glands - not collected for histopathology).

4. 90-R28 (6884-85). Female, adult, 8/22/90: **Appendix I (Continued)**

Vaccination area (SDM and tetracycline positive, antibody negative as of day 3).

Virus isolation: Tonsil - positive. All other tissues negative.

-Heart, diaphragm, tongue and M. muscle - all with a few granulomas (Hepatozoon).

-Lung - focal lipid pneumonia.

-Esophagus - a few Capillaria.

-(Tonsil - not collected for histopathology).

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5. 90-R25 (8505-06). Female, sub-adult, 8/23/90:

Vaccination area (SDM and tetracycline positive, antibody negative as of day 3).

Virus isolation: All tissues negative.

-Heart, diaphragm, tongue and M. muscle - all with a few granulomas (Hepatozoon).

-Intestine - few flukes (Phagicolla).

- cross-section of nematodes.

-Skin (body) - one focal area of dermatitis with mild focal sero-cellular crust over the epidermal surface and mild infiltrate in the subjacent dermis. (Recuts revealed cross-sections of ticks).

-Stomach - a few nematodes (Physoloptera).

-(Salivary glands, paw and tonsil - not collected for histopathology).

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6. 90-R21 (8207-08). Male, adult, 8/23/90:

Surveillance area (SDM and tetracycline negative, antibody negative as of day 3).

Virus isolation: All tissues negative.

-Intestines - with a few flukes (Phagicolla).

- (M. muscle, skin, paw, tonsil, salivary gland, prostate and urinary bladder-not collected for histopathology).

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7. 90-R31 (8009-10). Male, adult, 8/23/90:

Vaccination area (SDM and tetracycline positive, antibody negative as of day 3).

Virus isolation: All tissues negative.

-Heart, diaphragm, tongue, M. muscle, and salivary gland - all with a few granulomas (Hepatozoon).

-Pancreas - two flukes in duct (Eurytrema).

-Skin (paw) - many mites in hair follicles (Demodex). No cellular reaction.

-Lung - multifocal thromboemboli with cross-sections of degenerate nematode (Heartworm).

-(Tonsil - not collected for histopathology).

Appendix I (Continued)

8. 90-R29 (8513-14). Male, adult, 8/24/90:
Vaccination area (SDM and tetracycline positive, antibody negative as of day 4).
Virus isolation: Tonsil - positive. All other tissues negative.
-Heart, diaphragm, tongue and M. muscle - all with a few granulomas (Hepatozoon).
-Intestines - a few flukes (Phagicolla).
-Skin (face) - multifocal chronic ulcerative dermatitis (traumatic). GMS stains show a few fungi and some foreign bodies (plant material) within the reaction.
-(Tonsil - not collected for histopathology).

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9. 90-R32 (8116-17). Female, adult, 8/26/90:
Vaccination area (SDM and tetracycline positive, antibody negative as of day 6).
Virus isolation: All tissues negative.
-Heart, diaphragm, tongue, M. muscle - all with a few granulomas (Hepatozoon).
-Urinary bladder - mild diffuse cystitis.
-(Mesenteric lymph node and tonsil - not collected for histopathology).

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10. 90-R30 (8220-21). Male, adult, 8/31/90:
Vaccination area (SDM negative, tetracycline positive, antibody negative as of day 11).
Virus isolation: not completed to date.
-Heart, diaphragm, tongue and M. muscle - all with a few granulomas (Hepatozoon).
-Pancreas - one fluke in duct (Eurytrema).
-Intestines - a few flukes (Phagicolla).
-Skin - one focal dermal infiltrate (parasitic).
-(Tonsil - not collected for histopathology).

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11. 90-R26 (8327-28). Male, adult, 8/31/90:
Vaccination area (SDM negative, tetracycline positive, antibody negative as of day 11).
Virus isolation: All tissues negative.
All tissues of this animal, which had expired overnight following unsuccessful blood collection via the anterior vena cava, were partially autolyzed (owing to ambient temperatures in excess of 25^{o}C) and contained variable numbers of bacterial colonies. In all except the skin, there was no inflammatory cellular response, indicating severe tissue autolysis.
-Heart, diaphragm, tongue and M. muscle - all with a few granulomas (Hepatozoon).
-Tongue - a few Capillaria.

Appendix I (Continued)

-Lung - focal lipid pneumonia.
- moderate anthrocosis.

-Skin (body) - multifocal dermal thrombosis and vasculitis with many bacterial colonies (Gram positive cocci). There was also some multifocal dermal cellular infiltrate.

-Skin (paw) - lesions similar to above (body) present.

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12. 90-R27 (8623-24). Male, juvenile, 9/1/90:
Surveillance area (SDM and tetracycline negative, antibody negative as of day 12).
Virus isolation: Not completed to date.

-Heart, diaphragm and tongue - all with a few granulomas (Hepatozoon).

-Kidney - a small focal area of interstitial nephritis.

-Pancreas - fluke in duct (Eurytrema).

-Intestines - a few flukes (Phagicolla).

-Aorta - mild multifocal mineralizations in media.

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13. 90-R33 (8611-12). Male, adult, 9/1/90:
Vaccination area (SDM negative, tetracycline and antibody positive as of day 12).
Virus isolation: All tissues negative.

-Heart, tongue and M. muscle - all with moderate numbers of granulomas (Hepatozoon).

-Lung - focal lipid pneumonia.
- one focal area (grossly umbilicated) of chronic fibrosis.

-Intestines - some flukes (Phagicolla).

-Skin (body) - mild, multifocal mononuclear infiltrate in the superficial dermis and a small focal serocellular crust over the epidermis.

-Skin (paw) - multifocal areas of hyperplasia (acanthosis, hyperkeratosis and parakeratosis) present. Within these foci, acanthocytes show marked swelling and vacuolation, and in some basal cells there were eosinophilic intra-cytoplasmic inclusions (skin sections negative for viral particles by transmission electron microscopy and negative for cDNA of ERA rabies virus glycoprotein by PCR analysis; collected tissues negative for virus isolation by cell culture).

-Intestines - a few flukes (Phagicolla).

-Esophagus - a few Capillaria.

-(Diaphragm and tonsils - not collected for histopathology).

Appendix I (Continued)

14. 90-R22 (8607-08). Female, adult, 9/1/90:
Vaccination area (SDM negative, tetracycline positive, antibody negative as of day 12).
Virus isolation: Not completed to date.

-Heart, tongue and M. muscle - all with a few granulomas (Hepatozoon).
-Lung - mild, multifocal lipid pneumonia.
-Kidney - medulla with a few foci of tubular mineralization.
-Intestines - a few flukes (Phagicolla).
-(Brain was partially autolyzed).
-(Diaphragm and tonsil - not collected for histopathology).

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15. 90-R35 (8255-56). Female, adult, 9/3/90:
Vaccination area (SDM negative, tetracycline and antibody positive as of day 14).
Virus isolation: Not completed to date.

-Heart, diaphragm, tongue and M. muscle - all with a few granulomas (Hepatozoon).
-Tongue - with a few Capillaria.
-Lung - multifocal areas of lipid pneumonia with many eosinophils.
-Mesenteric lymph node - a few eosinophilic granulomas (parasitic).
-Skin (paw) - small focal areas of mild dermatitis.
-(Tonsil - not collected for histopathology).

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16. 90-R23 (8337-38). Female, adult, 9/3/90:
Vaccination area (SDM, tetracycline and antibody negative as of day 14).
Virus isolation: Not completed to date.

-Heart, diaphragm, tongue and M. muscle - all with granulomas (Hepatozoon).
-Lung - mild lipid pneumonia.
-Skin (body) - focal dermatitis and folliculitis with presence of bacterial colonies. Also, a few follicles with mites (Demodex).
-Skin (paw) - many mites (Demodex) in hair follicles. No cellular response.
-Stomach - mild, multifocal, eosinophilic granulomas and ulcerations (Physoloptera).
-(Brain - partially autolyzed).

Appendix I (Continued)

17. 90-R37 (8659-60). Male, adult, 10/14/90:

Surveillance area (SDM not done, first time capture was after day 14 of the trial when SDM testing was discontinued, tetracycline and antibody negative).

-Heart, tongue and M. muscle - all with a few granulomas (Hepatozoon).

-Intestines - with flukes (Phagicolla).

-Skin (paw) - mild multifocal epidermitis with bacteria.

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18. 90-R38 (8126-27). Male, adult, 10/14/90:

Surveillance area (SDM not done, tetracycline and antibody negative).

-Heart, diaphragm, tongue and M. muscle - all with a few granulomas (Hepatozoon).

-Pancreas - a few flukes (Eurytrema).

-Intestines - a few flukes (Phagicolla).

-Esophagus - with Capillaria.

-Skin (paw) - focal bacterial epidermitis.

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19. 90-R39 (8655-56). Male, adult, 10/15/90:

Vaccination area (SDM not done, tetracycline and antibody negative).

-Heart, diaphragm, tongue, and M. muscle - all with granulomas (Hepatozoon).

-Diaphragm, tongue and M. muscle - all with Sarcocystis.

-Lung - focal lipid pneumonia.

-Kidney - a few mineralized areas in medulla.

-Intestine - one deep ulceration with a nematode (thorny-head worm).

-Esophagus - with Capillaria.

-Skin (paw) - mild focal perifolliculitis.

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20. 90-R40 (8625-26). Female, adult, 10/15/90:

Vaccination area (SDM negative, tetracycline positive, antibody negative).

-Heart, diaphragm and M. muscle - all with granulomas (Hepatozoon).

-Skin (lower leg with wound) - severe, chronic, locally extensive cellulitis with many superficial bacterial colonies (probably traumatic).

-(Spleen, salivary gland, tonsil and ovary or uterus - not collected for histopathology).

Appendix I (Continued)

21. 90-R52 (Raccoon 6862-63). Male, adult, 11/26/90:
Vaccination area (SDM not done, tetracycline positive, antibody negative)
-Heart, diaphragm, tongue, M. muscle - all with granulomas (Hepatozoon).
-Skin (Head) - chronic-active dermatitis/cellulitis.

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22. 90-R56 (Raccoon 6870-71). Female, adult, 11/26/90:
Surveillance area (SDM not done, tetracycline and antibody negative),
-Kidney - mild multifocal tubular nephrosis.
-Skin (face - between the eyes) - ulcerated fibrotic area (old lesion).
-Intestines - with a few flukes (Phagicolla).
-Urinary bladder - with one large and a few small transitional cell papillomas.

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23. 90-54 (Raccoon 6823-24). Male, juvenile, 11/27/90:
Surveillance area (SDM not done, tetracycline and antibody negative)
-Skin (lower leg) - one focal ulceration and subjacent cellulitis.
-Intestines - a few flukes (Phagicolla).
-Mesenteric lymph node - a few parasitic granulomas.

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24. 90-R53 (Raccoon 8801-02/8576-77). Female, juvenile, 11/27/90:
Vaccination area (SDM and tetracycline positive, antibody negative)
-Heart, diaphragm, tongue, M. muscle - all with microgranulomas (Hepatozoon).
-Mesenteric lymph node - with a few parasitic granulomas.

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25. 90-R55 (Raccoon 8507-08). Male, juvenile, 11/27/90:
Vaccination area (SDM, tetracycline and antibody positive)
-Heart, diaphragm, tongue, M. muscle and stomach (smooth muscle) - all with granulomas (Hepatozoon).
-Pancreas - with flukes (Eurytrema).
-Intestines - with flukes (Phagicolla).

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26. 90-R57 (Raccoon 6820-22). Male, juvenile , 11/27/90:
Surveillance area (SDM not done, tetracycline and antibody negative)
-Pancreas - with flukes (Eurytrema).
-Intestines - with flukes (Phagicolla).

Appendix I (Continued)

27. 90-R63 (Raccoon 8857-8858). Female, subadult, 12/18/90:
Vaccination area (SDM not done, tetracycline positive, antibody negative)
-Gross findings: No gross lesions.
-Heart, diaphragm, M. muscle and tongue - all with moderate numbers of microgranulomata (Hepatozoon procyonis).
-Intestines - with flukes (Phagicola)

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28. 90-R64 (Raccoon 1699). Male, adult, 12/18/90:
Surveillance area (SDM not done, tetracycline and antibody negative as of 12/18/90)
-Gross findings: One Dirofilaria immitis within the heart.
-Heart and tongue - both with microgranulomas (Hepatzoon procyonis).
-Lungs - with multifocal areas of lipid granulomas.
-Skin (paw) - one small focal foreign body granuloma.
-Intestines - with flukes (Phagicola).

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29. 90-R65 (Raccoon 1700). Female, adult, 12/18/90:
Surveillance area (SDM not done, tetracycline and antibody negative as of 12/18/90)
-Gross findings: Several mm in diameter membranous lesion in the right ventricle near the apex which appears to be a congenital defect.
-Heart and diaphragm - both with (Hepatozoon procyonis) granuloma.
-Skin (body) - with one small focal foreign-body (hair) granuloma.
-Intestines - with flukes (Phagicola).

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30. 91-R7 (Raccoon 8836). Male, adult, 1/29/91:
Vaccination area (SDM not done, tetracycline positive, antibody negative as of 1/29/91)
-Gross findings: Swelling around prepuce and prominent inguinal lymph nodes as well as appearance of internal reproductive organs compatible with sexual activity. Small cyst in the cortex of one kidney, appears to be congenital.
-Heart, diaphragm and M. muscle - all with (Hepatozoon procyonis) granulomas.
-Tongue and tonsils - epithelium with Capillaria.
-Lung - with multifocal areas of lipid granulomas.
-Lung - with degenerate parasite (Heartworm) in arteries.
-Kidney - with two focal areas of chronic interstitial nephritis.
-Pancreas - with flukes (Eurytrema).
-Intestines - with flukes (Phagicola).

Appendix I (Continued)

-Skin (paw) - with mange (Demodex).
-Mesenteric lymph node - with parasitic (Phagicola) granulomas.

31. 91-R8 (Raccoon 0133-8056). Male, adult, 1/29/91:
Surveillance area (SDM and tetracycline negative, serology pending)
-Gross findings: Swelling around prepuce and prominent inguinal lymph nodes as well as appearance of internal reproductive organs compatible with sexual activity. Teeth worn to the gumline.
-Heart - with (Hepatozoon procyonis) granulomas.
-Lung - with multifocal lipid granulomas.
-Kidney - with a few small urinary retention cysts.
-Tonsil - epithelium with Capillaria.

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32. 91-R9 (Raccoon 6561-6562). Male, adult, 1/29/91:
Surveillance area (SDM not done, tetracycline and antibody negative)
-Gross findings: Swelling around prepuce and prominent inguinal lymph nodes as well as appearance of internal reproductive organs compatible with sexual activity. Integument within normal limits; superficial scratches on the neck.
-Heart, diaphragm, M. muscle, tongue, and intestines - all with moderate numbers of (Hepatozoon procyonis) granulomas.
-Tongue and esophagus - epithelium with Capillaria.
-Intestines - with flukes (Phagicola).
- Mesenteric lymph node - with focal parasitic granulomas.
-Tonsil - not present for histology.

BIOMARKER AND HISTOPATHOLOGIC FINDINGS,
OF NON-TARGET SPECIES

1. 90-R47 (Deer #1). Female, adult, 8/26/90:
Surveillance area (SDM and tetracycline negative)
-Heart, tongue, extra-ocular muscle and esophagus - all with a few Sarcocysts.
-Esophagus with a few Gongylonema parasites.
-Lung - with moderate numbers of lungworms.

(M. muscle, pancreas, lymph node, salivary gland, tonsils and thyroid not included for histopathology).

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2. 90-R48 (Deer #2). Male, adult, 8/29/90:
Surveillance area (SDM and tetracycline negative)

-Diaphragm, extra-ocular muscles and tongue with a few Sarcocysts.
-Lung with cross-sections of a few lungworms.
-Liver with cross-section of an unidentified parasite (subcapsular).
(M. muscle, spleen, lymph node, omasum, abomasum and urinary bladder not included for histopathology).

---

3. 90-R49 (Deer #3). Male, adult, 9/1/90:
Surveillance area (SDM and tetracycline negative)

-Diaphragm and tongue with a few Sarcocysts.
-Lung with cross-sections of a few lungworm larvae.
(M. muscle, pancreas, abomasum, salivary glands and tonsils not included for histopathology).

---

4. 90-R50 (Red Fox #1). Female, sub-adult, 8/23/90:
Surveillance area (SDM and tetracycline negative)

-Stomach - a few multifocal to focally extensive areas in the submucosa with macrophages.
(M. muscle, salivary glands, urinary bladder and tonsils not included for histopathology).

Table 1

Parramore Island

Raccoon Live-Trapping Summary

August 20, 1990 through January 31, 1991

	Vaccination Area			Surveillance Areas			Combined		
	New[1]	Recaptures	Total Captures	New	Recaptures	Total Captures	New	Recaptures	Total Captures
14 Days 8/20-9/3/90	100	74	174	26	8	34	126	82	208
30 Days 8/20-9/30/90	114	132	246	44	16	60	158	148	306
60 Days 8/20-10/31/90	134	197	331	55	30	85	189	227	416
90 Days 8/20-11/30/90	141	238	379	64	49	113	205	287	492
120 Days 8/20-12/31/90	146	242	388	75	67	142	221	309	530
150 Days 8/20-1/31/91	151	248	399	83	78	161	234	326	560

1 Individuals captured in the vaccination or surveillance areas since the initiation of the vaccine field trial.

Table 2

Parramore Island

Small Mammal Live-Trapping Grid Results

October 1990 through January 31, 1991

	Vaccination Area			Surveillance Areas			Combined		
	New[1]	Recaptures	Total Captures	New	Recaptures	Total Captures	New	Recaptures	Total Captures
October '90	61	7	68	16	1	17	77	8	84
November '90	87	112	199	65	36	101	152	148	300
December '90	31	106	137	55	71	126	86	177	263
January '91	14	33	47	7	15	22	21	48	69

1 Individuals captured in the vaccination or surveillance areas since the initiation of the vaccine field trial.

Table 3

Parramore Island
V-RG Vaccine Field Trial
Sulfadimethoxine Status versus
Virus Neutralizing Antibody (VNA) Status

Vaccination Area Resident Raccoons (N = 100)

		Rabies VNA Status		
SDM Status		Positive	NA[1]	Negative
Pos	38	10	20	8
Neg	11	4	5	2
Neg (Invalid)[2]	51	10	29	12
Total	100	24	54	22

Sulfadimethoxine (SDM) Biomarker Positive/Tested = 38/49 (77.5%)

Antibody positive/SDM positive = 10/18 (55%)

Seroprevalence of Rabies VNAs among residents 24/46 = 52%

---

1 Not available; raccoons have not been recaptured since day 14 of the trial.

2 SDM test negative but first capture was after day 6 of the trial when levels were expected to be declining.

Table 4

Parramore Island Radiotelemetry Summary

Through January 31, 1991

	Aug[1]	Sep	Oct	Nov	Dec	Jan
Radio-collars in the field at the begining of each month						
Raccoon	35	35	29	25	24[5]	23
Fox	5	5	4	4	4	4
Total	40	40	33	29	28	27
Successfully located						
Raccoon	35	29	25	22	22	20
Fox	5	4	4	4	4	4
Total	40	33	29	26	26	24
Collars removed from field						
Animal Died	0	0	2	0	0	1
Slipped[2]	0	6	1	1	1	0
Removed[3]	0	1	1	1	0	0
Total	0	7	4	2	1	1
Unable to locate during the month[4]	0	0	0	1	1	2

1 From the period August 20 through August 31, 1990.

2 Radio-collar signal was in mortality mode. Collar was recovered from the field after loss by the animal.

3 Radio-collar damaged, necessitating removal for refurbishment.

4 No signal from the radio-collar was received; researchers still actively searching for the animal.

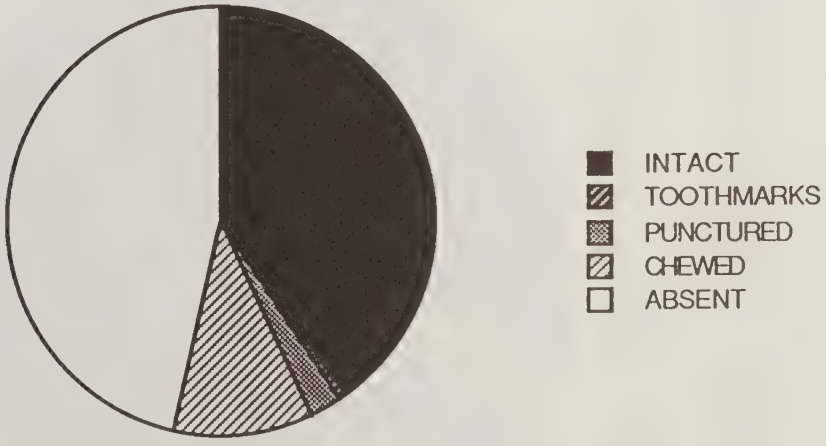
5 One additional raccoon was radio-collared.

FIGURE 1

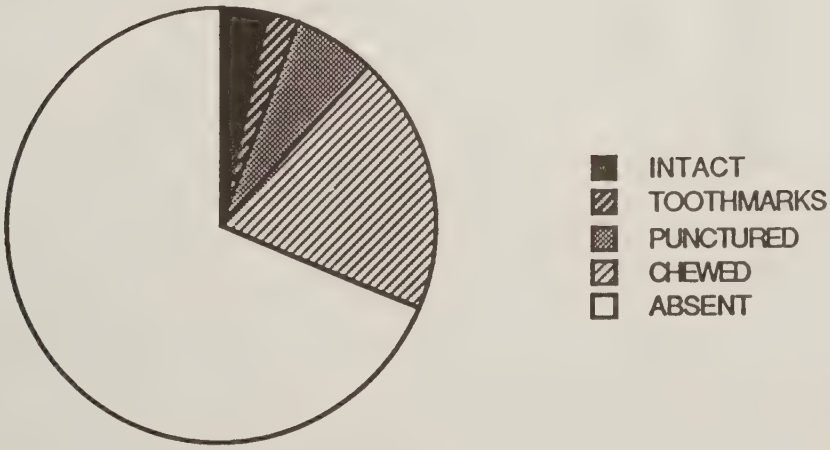
PARRAMORE ISLAND - V-RG VACCINE TRIAL

AMPULE CONTACT : DAY 1 (N=2924)

AMPULE CONTACT : DAY 3

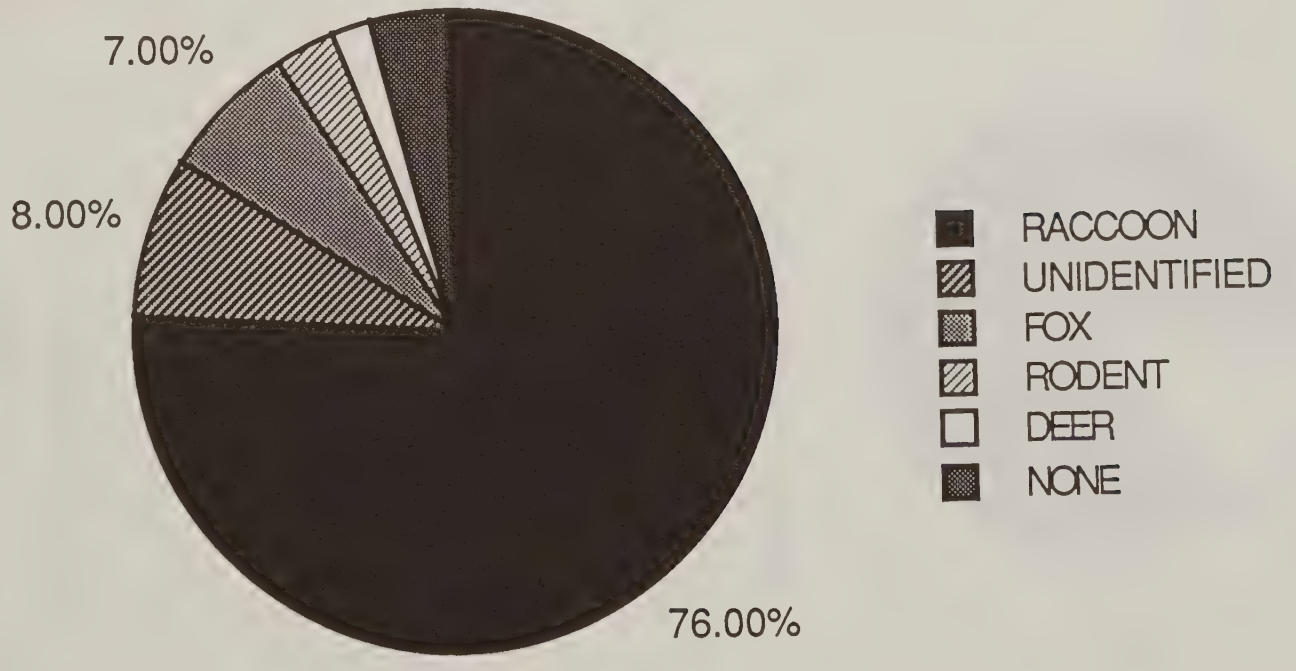


AMPULE CONTACT : DAY 5



PARRAMORE ISLAND - V-RG VACCINE TRIAL

TRACKING PIT DATA (N=100)



Development of a

Vaccinia-Rabies Glycoprotein Recombinant Virus Vaccine:

Safety Trials in Non-Target Species

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Development of a Vaccinia-Rabies Glycoprotein Recombinant Virus Vaccine: Safety Trials in Non-Target Species

Oral immunization of the red fox (Vulpes vulpes) is an important part of rabies control in Europe and Canada (15), (25), (29). While rabies control in the United States has been largely restricted to traditional measures, such as vaccination of dogs and cats, the intensity of the mid-Atlantic raccoon rabies outbreak has provided impetus for more comprehensive programs including oral wildlife immunization. Preliminary baiting trials have demonstrated that up to 70% of free-ranging raccoons (Procyon lotor) in rural areas of Pennsylvania accept placebo baits designed ultimately to deliver rabies vaccine (20). Although attenuated oral rabies vaccines result in successful immunization of foxes, they are ineffective by this route in raccoons or skunks (Mephitis mephitis) (3), (19), (23). Moreover, safety concerns exist over vaccine-induced disease in wildlife exposed to attenuated rabies viruses (12), (24). Thus, modified-live rabies virus vaccines do not satisfy the need for safe and efficacious oral rabies vaccines suitable for the most important North American wildlife vectors. A vaccinia-rabies glycoprotein recombinant vaccine has proven to be an effective oral immunogen in raccoons (31), (19) and a variety of other species (21), (11), providing long-term protection against rabies (22), (6). The advantages of this recombinant orthopox rabies vaccine include greater thermostability than attenuated rabies vaccines, maximum duration and efficacy of a live virus vaccine system, and the inability to cause rabies, since only the cDNA of the surface glycoprotein of the Evelyn-Rokitnicki-Abelseth (ERA) strain of of rabies virus is included in the recombinant virus (16).

In the United States, bait development and placebo baiting trials have been conducted in an attempt to tailor the vaccine packaging and distribution methods to be specifically attractive to raccoons and less attractive to other species (20). Nevertheless, when vaccine is offered as a free choice in baits under natural conditions, contact by non-target wildlife species cannot be totally excluded by bait design and distribution alone. A rational, methodical approach is needed to evaluate potential V-RG vaccine safety in the array of non-target species potentially at risk for

vaccine contact. Relevant major taxonomic groups were defined as those most likely to contact vaccine-laden baits under natural conditions. Biomarkers were used to determine these groups in placebo trials in the United States (14), (13) and actual oral rabies vaccination campaigns in Europe and Canada (18), (25), (29). Relevant groups included ecological competitors of raccoons and foxes, such as the opossum, mustelids, other members of the Canidae family, and rodents. Additionally, other species were included because of their association as companion animals with humans, for example, dogs and cats; domestic livestock, such as cattle and sheep; or commonly harvested game species such as white-tailed deer. The rationale for including avian species was based partially upon observations by Canadian researchers (2) indicating significant bait interference by crows, although this phenomenon has not been observed with a fishmeal polymer bait intended for use with raccoons in the United States (Rupprecht, unpublished data). Lastly, vaccine safety testing was conducted in members of the avian orders Falconiformes and Strigidae because of the possibility of indirect vaccine exposure through consumption of animals that had recently contacted vaccine. The selected array of non-target species in which V-RG vaccine safety evaluations were conducted was not exhaustive; rather, it was designed to represent those species in close contact with humans or wildlife species at highest risk for bait contact.

The overall objectives were to conduct V-RG vaccine safety evaluations in as broad a range of taxonomic groups as possible rather than in maximal numbers of a few limited species. The husbandry of exotic species involves many unknowns, such as various nutrient requirements, adaptation to commercial feeds, effects of confinement, artificial housing and environment, and presence of humans and conspecifics, which can adversely affect viable numbers available for vaccine testing. For example, some researchers (1) demonstrated that several of their experimental voles and 11 of 13 gulls from both the control and V-RG vaccine groups expired during the course of the experiment from diseases unrelated to V-RG virus administration. In their gulls, postmortem examination revealed heavy infestations of one or more parasites (microfilaria, kidney nematodes, renal coccodiosis) and two were infected with Salmonella typhimurium; histopathology did not reveal any lesions consistent with vaccine virus infection. Thus, the number of exotic

species tested may be limited by the number available from the wild and their successful adaptation to captive conditions. Unsuccessful adaptation to captivity may preclude certain species from safety testing in confinement.

Additionally, unlike inbred strains of laboratory animals, a homogenous population of each species was not available for experimental use. The age, sex, body condition, and source of animals was often varied for V-RG vaccine safety testing. A cross-sectional representation of wildlife populations was selected: ages ranged from weanlings to aged individuals; body condition varied from good to nearly emaciated; and parasite burdens were minimal to severe. These animals were not necessarily in prime condition, as normally expected in laboratory animal stock. For example, one deer was an amputee and some wild kestrels and hawks had suffered crippling injuries in the wild. Additionally, captive conditions are a source of stress upon previously free-ranging individuals. These combined factors provided rigorous conditions in which to test vaccine safety. If untoward effects were to be frequently expected in field applications of vaccine, some indication of the potential for harmful effects due to the vaccine should have been observed in the individuals experiencing these adverse conditions of captivity.

When available, animals were obtained from commercially available sources or live trapped from the wild. Water and food were offered *ad libitum*. Housing and husbandry was in accordance with each individual Canadian or European investigators' local guide to the care and use of laboratory or exotic animals. Practices in the USA conformed both to the USDA and NIH guidelines. Common sedatives such as ketamine hydrochloride were used regularly when appropriate to minimize the stress of handling and to ensure the safety of personnel.

The V-RG recombinant virus vaccine was stored lyophilized at 4^{o} C prior to use. It was reconstituted in sterile distilled water, pH 7.4, and was either used in undiluted form ($10^{9.0}$ pfu/ml) or was diluted in a 20% PBS solution to the required concentration for the experiments cited (Table 1). All animals were sero-negative for rabies virus neutralizing antibodies (VNA) prior to the studies. Specific protocols, as to titration of VNA, chemical sedation, euthanasia method, and necropsy were as described in the references or, if unpublished, were similar to those previously

detailed for raccoons (22). The numbers of species tested, observation periods, rabies-specific VNA (measured between days 14 and 28 depending upon experimental protocol) and vaccine titers administered are listed in Table 1.

The V-RG recombinant virus vaccine has been evaluated in the laboratory for safety in over 40 warm blooded vertebrate species, primarily by the oral route of administration but also by the intradermal, intramuscular, subcutaneous, intestinal, ocular, and intranasal routes, as potential accidental routes of inoculation in the wild. For example, the intramuscular or subcutaneous routes mimic the bite of a conspecific after consuming a vaccine-laden bait; intradermal introduction simulates an animal's licking abrasions immediately after contacting vaccine. There has been no vaccine-associated morbidity or mortality and no gross pathological lesions observed in over 320 individual animals representing some 20 taxonomic families. With rare exceptions, there has been no demonstration of generalized contact-transfer of vaccine between vaccinated and control animals housed together (5), (22). Moreover, in vaccine pathogenicity studies, virus was limited to selected tissues, such as tonsils, buccal mucosa, and retropharyngeal lymph nodes, within a limited time period (21), (26). Taken as a whole, these extensive laboratory safety experiments have documented the innocuity of V-RG vaccine in all species tested to date, as well as its effectiveness as an oral immunogen against severe street rabies virus challenge in a majority of the species tested. The logical extensions of laboratory safety evaluations are limited field trials of the vaccine to evaluate its safety in complex natural ecosystems.

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Table 1

Summary of
Vaccinia-Rabies Glycoprotein Recombinant Virus Vaccine Safety Trials in Non-Target Species[1]

Species	Number[2]	Dose(pfu)[3]	Route	VNA[4] (range, IU/ml)	Observation Period (days)	Reference
CLASS MAMMALIA						
Order Marsupiala						
Family Didelphidae						
Opossum (Didelphis virginianus)	6	10^7	oral	0.9 - 56.8	30	(21)
Order Carnivora						
Family Canidae						
Red Fox	2	10^8	oral	ND	0.5	(26)
(Vulpes vulpes)	2	10^8	oral	"	1	
	2	10^8	oral	"	2	
	1	10^8	oral	"	4	
	10	10^6	oral	0.1 - 2.3	28	(5)
	10	10^7	oral	0.1 - 24.6	"	
	10	10^8	oral	0.1 - 6.7	"	
	8	$10^{8.5}$	bait	11.1 - 44.5	90	(28)
	6	$10^{8.5}$	GI	1.4 - 5.6	"	
	2	$10^{8.5}$	ID	22.3	"	

Species	Number[2]	Dose(pfu)[3]	Route	VNA[4] (range, IU/ml)	Observation Period (days)	Reference
Family Canidae (cont.)						
Red Fox	2	10^8	ID	2.6 - 3.0	28	(5)
(Vulpes vulpes)	2	10^8	SC	0.1 - 3.0	"	
	4	10^8	oral +	1.9 - 2.7	"	
	4	10^8	oral	0.1 - 24.6	365	(6)
	4	10^4	oral	0.1 - 1.3	28	(6)
	4	10^6	oral	0.1 - 0.8	"	
	4	10^8	oral	2.3 - 2.7	"	
	5	10^8	bait	1.1 - 2.6	"	
	2	$10^{7.6}$	ocular	> 0.5 (2/2)	30	(11)
	2	$10^{7.6}$	intranasal	> 0.5 (2/2)	30	
Fox Cubs (V. vulpes)	13	$10^{7.2}$	oral	0.1 - 28.9	33-365	(8)
Dog	3	$10^{4.6}$	SC	0.1 - 5.1	28	(6)
(Canis familiaris)	3	$10^{6.6}$	SC	2.1 - 9.6	"	
	3	$10^{8.6}$	SC	1.9 - 14.0	"	
	4	$10^{8.6}$	oral	0.1 - 3.6	28	(6)
	4	$10^{9.6}$	oral	1.6 - 13.7	"	
Grey Fox (Urocyon cinereoargenteus)	3	10^9	oral	486.0 - 1458.0	30	Wistar, unpub.
Coyote (Canis latrans)	10	$10^{7.9}$	bait	0.17 - 5.6	90	(1)

Table 1 (cont.)

Species	Number[2]	Dose(pfu)[3]	Route	VNA[4] (range, IU/ml)	Observation Period (days)	Reference
Family Felidae						
Bobcat (Lynx rufus)	3	10^9	oral	0.3 - 486.0	30	Wistar, unpub.
Cat (Felis domesticus)	4	10^8	oral	0.1 - 39.0	115	(6)
	3	10^4	SC	0.1	52	(6)
	3	10^6	SC	2.5 - 17.4	"	
	3	10^8	SC	2.4 - 27.0	"	
Family Mustelidae						
Skunk	8	10^9	bait	0.1 - 4.61	90	(27)
(Mephitis mephitis)	8	10^9	GI	0.1- 14.4	"	
	6	$10^{8.3}$	ID	44.5 - 159.0	"	
	3	$10^{8.3}$	IM	19.6 - 37.4	"	
River Otter (Lutra canadensis)	3	10^9	oral	6.0 - 18.0	30	Wistar, unpub.
Mink (Mustela vision)	7	$10^{7.7}$	oral and ID	6.0 - 162.0	180	Wistar, unpub.
Ferret	2	10^8	oral	1.3 - 15.3	28	(7)
(Mustela putorius)	2	10^9	oral	3.2 - 15.3	"	
European Badger (Meles meles)	6	$10^{8.3}$	oral	6.1 - 68.8	45	(10)
Family Ursidae						
Black Bear (Ursus americanus)	3	$10^{8.8}$	oral	≤0.2	30	Wistar, unpub.

Species	Number[2]	Dose(pfu)[3]	Route	VNA[4] (range, IU/ml)	Observation Period (days)	Reference
Order Artiodactyla						
Family Bovidae						
Cattle	10	10^8	SC	4.9 - 40.0	120	(17)
(Bos taurus)	10	10^8	ID	4.6 - 40.0	"	
	2	10^8	ID	12.8 - 20.3	35	(7)
	1	10^8	SC	17.6	"	
	1	10^8	IM	1.1	"	
Sheep (Ovis ovis)	4	10^7	oral	> 0.5 (4/4)	30	(4)
Family Suidae						
Wild Boar (Sus scrofa)	4	$10^{8.3}$	oral	0.5 - 5.5	88	(10)
Family Cervidae						
White-Tailed Deer (Odocoileus virginianus)	4	10^9	oral	54.0 - 1458.0	30	Wistar, unpub.
Order Lagomorpha						
Family Leporidae						
European Rabbit (Oroctolagus sp.)	4	$10^{8.3}$	ID	>444.4	14	(30)
	2	$10^{7.8}$	ID	126.0	21	(31)
	2	"	IM	711.1	"	
	2	"	SC	1037.0	"	
	2	"	oral	1037.0	"	
	3	$10^{7.6}$	ID	0.1 - 177.8		(31)
		$10^{7.6}$	ID[5]	355.5 - > 1037.0	180	

Table 1 (cont.)

Species	Number[2]	Dose(pfu)[3]	Route	VNA[4] (range, IU/ml)	Observation Period (days)	Reference
Order Rodentia						
Family Muridae						
House Mouse	12	$10^{8.3}$	ID	>444.4	14	(31)
(Mus musculus)	12	$10^{7.7}$	FP	>444.4	"	
	6	$10^{6.9}$	oral	18.5 - 444.4	14	(22)
	6	$10^{6.9}$	oral+	>444.4	"	
Family Erethizonidae						
Porcupine (Erethizon dorsatum)	3	$10^{9.0}$	oral	162.0 - 1458.0	30	Wistar, unpub.
Family Sciuridae						
Groundhog (Marmota monax)	10	$10^{7.9}$	oral	11.0 - 89.0	90	(1)
Grey Squirrel (Sciurus carolinensis)	11	$10^{7.9}$	oral	0.4 - 22.0	90	(1)
Family Cricetidae						
Cotton rat	1	10^{8}	oral	ND	1	Wistar, unpub.
(Sigmodon hispidus)	1	"	"	"	2	
	1	"	"	"	3	
	1	"	"	"	4	
	4	"	"	0.3 - 486.0	30	
Marsh Rice Rat (Oryzomus palustris)	7	10^{8}	oral	0.2 - 18.0	60	Wistar, unpub.
Syrian Hamster (Mesocricetus auratus)	12	10^{7}	IM	> 0.5 (12/12)	30	(31)

Species	Number[2]	Dose(pfu)[3]	Route	VNA[4] (range, IU/ml)	Observation Period (days)	Reference
Family Cricetidae (Cont.)						
Field Vole (Microtus agrestis)	1	$10^{6.5}$	oral	>0.5 (1/1)	35	(10)
Meadow Vole (Microtus pennsylvanicus)	12	$10^{7.9}$	oral	0.1 - 22.3	30	(1)
	14	10^{9}	oral	0.7 - 44.0	90	
Common Vole (Microtus arvalis)	2	$10^{6.5}$	oral	>0.5 (2/2)	35	(10)
Bank Vole (Clethrionomys glareolus)	13	$10^{6.3}$	oral	>0.5 (8/13)	35	(10)
Water Vole (Arvicola terrestris)	5	$10^{6.5}$	oral	>0.5 (4/5)	35	(10)
Deer Mouse (Peromyscus maniculatus)	10	$10^{9.0}$	oral	0.7 - 18.0	90	Wistar, unpub.
European Field Mouse (Apodemus sp.)	4	$10^{6.3}$	oral	>0.5 (3/4)	41	(10)
Yellow-Necked Mouse (Apodemus flavicollis)	7	$10^{6.5}$	oral	>0.5 (5/7)	41	(10)
Wood Mouse (Apodemus sylvaticus)	27	$10^{6.3-6.5}$	oral	>0.5 (16/27)	28-43	(10)

Table 1 (cont.)

Species	Number[2]	Dose(pfu)[3]	Route	VNA[4] (range, IU/ml)	Observation Period (days)	Reference
CLASS AVES						
Order Falconiformes						
Family Accipitridae						
Red-Tailed Hawk (Buteo jamaicensis)	6	10^8	oral	0.3 - 14.0	30	(1)
Kestrel (Falco tinnunculus)	4	10^8	oral	0.1	30-45	(10)
Carrion Crow (Corvus corone)	17	10^8	oral	0.1	28	(10)
Common Buzzard (Buteo buteo)	8	10^8	oral	0.1	30-45	(10)
Order Charadriiformes						
Family Laridae						
Ringbill Gull (Larus delawarensis)	2	$10^{7.9 \text{ or } 8.1}$	oral	0.1 - 1.4	90	(1)
Order Strigiformes						
Family Strigidae						
Great Horned Owl (Bubo virginianus)	8	10^8	oral	0.2 - 0.7	30	(1)

Species	Number	Dose(pfu)[2]	Route[3]	VNA[4] (range, IU/ml)	Observation Period (days)	Reference
Order Passeriformes						
Family Grallinidae						
Magpie (Pica pica)	7	10^8	oral	0.1	28	(10)
Family Corvidae						
Jay (Garrulus glandarius)	2	10^8	oral	0.1	28	(10)

1 Animal experiments were conducted from 1983 to 1990.

2 PFU = plaque forming unit = tissue culture infectious dose.

3 Oral = vaccine administered via needle-less syringe directly onto the tongue: Oral + = scarification of the oral cavity and deposition of vaccine; ID = intradermal; SC = subcutaneous; IM = intramuscular; GI = gastrointestinal deposition via endoscope; FP = footpad; Bait = vaccine offered free choice in bait.

4 Rabies-virus neutralizing antibody (VNA) expressed in international units per milliliter, determined by a rapid fluorescence focus-inhibition test or fluoresence inhibition microtest. ND = not done. Range given, if available, otherwise sero-conversion is indicated by > 0.5 IU/ml followed by number sero-positive over total number in group (in parenthesis).

5 Second (booster) dose administered 6 months after primary dose.

Development of a Vaccinia-Rabies Glycoprotein Recombinant Virus: Further Characterization of Vaccine Safety in Primates

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April 15, 1991

Development of a Vaccinia-Rabies Glycoprotein Recombinant Virus Vaccine: Safety Evaluation in Primates.

Over the last decade, research has progressed in Europe and North America on the control of wildlife rabies via oral vaccination of free-ranging carnivores by means of vaccine-laden baits [1-6]. Although attenuated rabies virus vaccines may be relatively safe and effective for red fox *(Vulpes vulpes)* rabies control, modified-live viruses may not be completely effective or innocuous for all species [7,8]. The development of a vaccinia-rabies glycoprotein (V-RG) recombinant virus vaccine [9-10], demonstrated to be efficacious against lethal street rabies virus infection, has the added advantage of the inability to cause vaccine-induced rabies. Laboratory trials of the V-RG vaccine virus in major vector species [11-16], such as the raccoon *(Procyon lotor)*, red fox and striped skunk *(Mephitis mephitis)*, have demonstrated the safety of the vaccine, regardless of route or dose, even in juvenile, pregnant or lactating animals. In addition, the innocuity of the V-RG vaccine has also been established for more than 40 different non-target animals, including opossums *(Didelphis virginianus)*, coyotes *(Canis latrans)*, woodchucks *(Marmota monax)*, grey squirrels *(Sciurus carolinensis)*, meadow voles *(Microtus pennsylvanicus)*, red-tailed hawks *(Buteo jamaicensis)*, gulls *(Larus delawarensis)*, great-horned owls *(Bubo virginianus)* and a wide variety of other wild and domestic North American and European birds and mammals [17-21].

Regulatory requirements pertaining to the purity and potency evaluation of a V-RG recombinant virus vaccine, specifically intended for the oral vaccination of free-ranging carnivores against rabies, are relatively clear when compared to the larger complexities of overt vaccine safety once released in an ecosystem, considering the myriad of non-target species that may be inadvertently exposed to recombinant virus, including humans. As a major safety advantage, the V-RG vaccine has not been shown to be pathogenic, oncogenic, teratogenic, or result in biological transmission to conspecifics. These clinical laboratory observations, conducted independently by American, Canadian and European investigators, have recently been corroborated by widespread field trials on the European mainland and a limited but intensive island trial [22] in the United States, with no untoward effects detected.

However, no existing veterinary biological in current use can be considered as completely "risk-free" to every conceivable life history category for all species. With this in mind, baits and baiting strategies for oral rabies vaccination are purposefully designed to minimize deliberate exposure to domestic animals and humans [22]. In addition, public education and carefully coordinated biosecurity measures, selective temporal and spatial distribution methods, warning labels, and the addition of malodorous substances repugnant to humans, have been designed as public health safeguards. Nevertheless, questions frequently arise concerning the potential

outcome resulting from accidental human exposure to an animal that had recently consumed a V-RG recombinant virus vaccine-laden bait.

Additionally, although the V-RG recombinant vaccine is considered a veterinary biological, with the eventual intended purpose of controlling rabies through the induced herd immunity in free-ranging carnivores by oral vaccination, certain public health issues remain, pertaining to accidental human V-RG viral exposure. It is considerably easier to demonstrate the relative merits and inherent safety of a given biological for its intended animal hosts by direct experimentation, while perceived secondary effects to non-target species, such as humans, are considerably more difficult to evaluate. In the last decade, with the field deployment of more than 10 million doses of live rabies virus vaccine in Western Europe, [4,5] less than a handful of human exposures to vaccine-laden baits have occurred. Thus, exposure to vaccine is expected to be limited; by conceptual extension of safety tests from other mammals, similar in comparative anatomy and physiology, the V-RG vaccine should also be relatively innocuous to humans should exposure occur.

In the further consideration of future field trials of the recombinant vaccine on the North American mainland, primates were tested in captivity for an additional safety assessment of the V-RG virus. The objective of these experimentswas to evaluate the clinico-pathological outcome of deliberate transdermal and mucosal inoculation of the recombinant virus in a primate model, in order to mimic either indirect human exposure (i.e., occurring via bite from a recently vaccinated animal) or direct accidental traumatic exposure (i.e., to a vaccine-laden bait), to the V-RG virus.

In the first experiment, primates consisted of 26 adult squirrel monkeys, *(Saimiri sciureus)*, represented by both sexes. Weight was within the normal range for the sex and age of the species. Each animal was identified by a unique tattoo and was housed individually in stainless-steel primate squeeze cages. Monkeys were fed monkey chow once daily in the afternoon and fresh produce once daily in the morning. Fresh water was available *ad libitium* via self-dispenser. Room temperature was maintained at $25^{o} \pm 5^{o}C$ and relative humidity was ambient. A 12-hour light-dark cycle was provided. Animals were quarantined for four weeks prior to study initiation and were in good health as determined by a staff veterinarian.

On day 0, monkeys were randomly selected from an available pool of animals and eight each were stratified to one of three experimental groups. To mimic an animal bite, vaccinia virus vaccine was administered (10^{8}pfu/ml) by intradermal scarification of a shaven area of the dorsal aspect of the upper torso, using a bifurcated stainless-steel needle in a manner similar to that previously used for routine smallpox scarification of humans. The vaccinia virus strains consisted of either the V-RG recombinant virus vaccine, the parental strain of the V-RG virus used for recombinant vaccine construction, or the New York Board of Health virus vaccine. Two control animals received only phosphate buffered saline (PBS) medium in lieu of vaccinia virus.

Twice daily, each animal was examined for evidence of abnormal physical or behavioral traits. Special attention was given to local lesions or secondary site lesion development (e.g., mucosal areas). On day seven after inoculation, and weekly thereafter, 0.2 ml of blood was collected for the analysis of VNA titers. Oral and fecal swabs were also collected at this time for later virus isolation. Samples were preserved in Eagles minimal essential medium plus 10% fetal calf serum with penicillin and streptomycin and individually stored frozen at -70°C until analysis. Necropsies were conducted on all animals which became moribund or which died during the study. Scheduled necropsies included one control animal and two animals from each test group, which were euthanatized at day seven after inoculation. In addition, two animals per experimental test group were each euthanatized on day 14 and on day 21 of the study. The remaining six experimental animals and one control monkey were euthanatized on day 60 of the study. Animals were euthanatized via barbiturate overdose. A complete gross necropsy was performed at that time.

No squirrel monkeys demonstrated evidence of abnormal physical or behavioral traits, regardless of group assignment. No secondary (e.g., mucosal) lesions developed and no vaccinia virus was isolated from oral or fecal swabs. Within three to five days post-vaccination, the inoculation sites of all monkeys became slightly indurated; moderate local inflammation progressed in all groups, except the PBS-controls. No significant difference in mean lesion diameter was observed, regardless of group assignment (Table 1). However, when examined clinically and at necropsy, dermal erosions were noted in the parental and NYBH vaccinia virus groups in seven to 14 days, whereas in the V-RG group, lesions were confined to the epidermis, at the local level of infiltration. Despite these changes between groups, all local lesions had begun to heal within three weeks and in the following observation period. No remarkable alterations in any organ were observed at gross necropsy and no viral recrudesence or adverse clinical signs were observed in monkeys held until day 60. Rabies VNA were detected at seven to 14 days from seven of the eight monkeys receiving the parenteral V-RG virus vaccine, but not in other experimental groups.

In another set of experiments, primates consisted of colony-born chimpanzees, *(Pan troglodytes)*, greater than two years of age. All chimpanzees weighed within the normal range for their age and sex, and were identified by a unique tattoo. Animals were fed fresh produce once daily in the morning and Purina monkey chow, once daily in the afternoon. Water was available *ad libitum* via self-dispenser. Chimpanzees were housed in individual cages in well ventilated, air-conditioned rooms maintained between 72° and 82° F; humidity was ambient. A 12-hour light/dark cycle was provided. Animals were randomly selected for the experiment from an available pool of chimpanzees. Considering the relative scarcity of chimpanzees for experimental purposes, one animal was included in the trial that otherwise would not have been considered for routine vaccination. This particular chimp had a history of dermatitis, with small scabs present on

the skin of her upper extremities, which was provisionally diagnosed from bacterial culture as a chronic *Staph aureus* infection.

On day 0, eight experimental chimpanzees were given 1.0 ml of the V-RG recombinant virus vaccine ($10^{7.2}$ pfu/ml) orally via either needleless syringe or a polyethylene dosing cup (self-administered). Three sentinel animals were held in the same room in separate cages but were not given vaccine. Animals were not prevented from the free exchange of saliva (i.e., spittle), feces or other body fluids between cages during the experiment. After vaccine administration, every animal was closely observed for any adverse clinical signs or lesions. Animals were bled on days 0, 14 and 30 for potential anti-rabies VNA development. Fresh fecal samples were obtained from each animal, daily for the first week, twice during week two, and once during weeks three and four for potential V-RG virus isolation. Oral swabs were obtained on days 14 and 30. Serum and swab samples were stored frozen prior to analysis, at -20° and -70°C, respectively.

A related trial consisted of a group of 14 chimpanzees. Husbandry was the same as detailed for the first chimpanzee experiment. Animals consisted of the eight original chimpanzees receiving V-RG vaccine per os in the first experiment, three new naive experimental animals, and three unvaccinated sentinel animals held in the same room as the vaccinates. All animals were caged individually. On day 0 (66 days after the first administration of V-RG recombinant virus vaccine from experiment one), all experimental chimpanzees (excluding the 3 control sentinels) were given 1.0 ml of V-RG recombinant virus vaccine ($10^{9.0}$ pfu/ml) per os, either by needleless syringe or via a disposable polyethylene dosing cup.

Thereafter, each animal was observed twice daily for evidence of abnormal physical or behavioral traits. Special attention was given to potential oral lesions or secondary site lesion development. Fecal samples were obtained once daily from each animal for the first week; one sample from each animal twice weekly during the second week; and one weekly sample from each animal for the third and fourth weeks. Oral swabs were taken on days 14 and 30, and all swabs were stored as above. A 2.0 ml serum sample was collected from each animal on days 14 and 30, and was stored frozen at -20°C until analysis. Fecal, oral, and blood samples were taken more frequently from any clinically abnormal animals, as necessary. Necropsies were conducted on all animals that became moribund or died during the conduct of the study. All personnel in direct contact with the study animals for both experiments one and two were bled on days 30 and 60 for potential rabies VNA development.

In the first chimpanzee experiment, no V-RG recombinant virus was isolated from either oral or fecal swabs. No VNA titers were detected in either the experimental or sentinel chimps over the 30 days of observation All animals remained completely healthy throughout the study.

Considering the poor immunological response via oral V-RG virus vaccination in experiment one, from the failure of chimpanzees to develop detectable VNA from the administered

dose, some two months later animals were administered a second oral dose, amounting to a two log increase in concentration. This would approximate a dose resulting from the consumption of at least ten vaccine-laden baits from the field, assuming a minimal individual dose of $10^{8.0}$ pfu/ml per bait deployed for raccoon rabies control [11]. The induction of VNA in these chimps is demonstrated in Table 2.

No vaccine virus was recovered from any of the oral and fecal swabs which were collected from the chimpanzees, with the exception of a single case, where vaccinia virus was isolated from an oral swab on the 6th day after vaccination from the chimpanzee which suffered from bacterial dermatitis. Apparently, at the same time the animal had a low grade fever, lack of appetite and slightly enlarged local lymph nodes. Treatment with antibiotics (cephalexin, 1.0 gm/day for five days) and with Tylenol cleared the animal completely of any clinical signs and it remained healthy to date, except for the previously observed bacterial dermatitis. No virus was recovered from stool samples and apparently transient clinical signs were attributed to the slight recrudescence of pre-existing bacterial infection. No virus was detected in oral or fecal swabs obtained from the other 10 experimental chimpanzees. Six of the animals developed a high titer of VNA and two had a relatively low titer. The antibody titers remained at the same level for at least one month. Control sentinel chimps and human animal caretakers remained sero-negative, despite close physical contact from daily husbandry duties.

The relative innocuity of the V-RG vaccine, even when administered at high doses directly to mucosal surfaces, has been demonstrated in these and numerous other independent animal models under fairly severe experimental conditions. It was previously established that inactivation of the thymidine kinase gene of vaccinia virus by the insertion of a c-DNA sequence coding for foreign genes would greatly attenuate vaccine viral virulence [23]. For example, in outbred laboratory mice, the intra-cerebral LD_{50} dose of V-RG was estimated to be in excess of $10^{9.0}$ pfu/ml [20]. Therefore, considering what is already known of human response to the parental vaccine, accidental exposure to the attenuated V-RG virus is not expected to produce any severe complications.

Taken together, these experimental and epidemiological data demonstrate the marked attenuation of the V-RG recombinant virus. No adverse effects of the vaccine have been demonstrated in any wildlife that might come in contact with vaccine-laden baits [11, 13-21]. Selective bait formulation and deployment will maximize contact by target species (raccoons) and minimize exposure to non-target animals [22]. Careful attention to site selection, biosecurity and baiting strategy should greatly decrease the probability of human or domestic animal contact. If human exposure to vaccine should unexpectedly occur, the clinical outcome will quite likely be modified by the specific exposure route and number of doses of vaccine contacted. Deliberate experimental oral exposure of primates to high concentrations of the V-RG vaccine has shown that

it is extremely unlikely that adverse clinical signs or lesions will result. Additionally, if indirect transdermal exposure should occur via bite of a recently vaccinated animal, the consequences specific to the V-RG vaccine will depend in part upon the dose(s) that were consumed by the animal and the time delay between bait consumption and bite incident itself, because residual virus in the animal's mouth is minimal and subject to a relatively rapid clearance [12]. Purposeful inoculation of the V-RG virus via scarification of primate skin (mimicking an animal bite) has also shown that the clinico-pathological prognosis would be extremely favorable even after exposure by an animal recently consuming vaccine. Thus, with these additional safety data in hand, future mainland trials of recombinant vaccine should be strongly encouraged in North America and elsewhere, in order to properly characterize the dynamics of the V-RG virus under actual field conditions.

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Table 1

Comparative Pathogenecity of Vaccinia and Vaccinia-Recombinant Viruses: Intradermal Scarification (10^8 pfu/ml) in Squirrel Monkeys

Groups	x ± SE (mm) Lesion Diameter (Sample Size)		
	D 7	D 14	D 21 ff
NYBH	2.1 ±0.6 (N=7) (dermal erosions-dermal ulcerations)	5.0 ± 2.5 (N=5) (pinpoint-dermal ulcerations)	Scars-no detectable lesions
VACC-COP.	2.4 ± 0.4 (N=8) (dermal erosions-dermal ulcerations)	2.0 ± 0.4 (N=5) (pinpoint-dermal ulcerations)	Scars-no detectable lesions
V-RG	2.5 ± 0.3 (N=8) (pinpoint-epidermal erosions)	2.0 ± 0.5 (N=6) (pinpoint-epidermal erosions)	Scars-no detectable lesions

Table 2

Development of rabies virus neutralizing antibodies in chimpanzees given a vaccinia-rabies glycoprotein recombinant virus vaccine ($10^{9.0}$ pfu/ml) per os.

Animal #	Rabies VNA Titer			
Previous Vaccinates	Day 0	Day 9	Day 14	Day 28
47	<5	-	405	405
76	<5	-	45	45
88	<5	-	<5	<5
49	<5	135	135	405
116	<5	-	135	45
132	<5	-	<5	<5
133	<5	-	15	135
48	<5	-	405	135
New Vaccinates				
121	<5	-	135	1215
127	<5	45	45	135
147	<5	-	<5	<5
Sentinel Controls				
78	<5	-	<5	<5
122	<5	-	<5	<5
134	<5	-	<5	<5

Development of a Recombinant Rabies Vaccine: Immunodeficient Host Studies

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Pathogenesis of V-RG virus in immunodeficient animals

Following initial efficacy studies in the development of a vaccinia-rabies glycoprotein (V-RG) recombinant virus vaccine, considerable attention was next focused upon the experimental safety and pathogenesis of the virus in a number of species by a variety of different routes of administration. These studies demonstated that limited amounts of the V-RG virus could be isolated from local tissues, such as tonsils and sub-mandibular lymph nodes, of immuno-competant raccoons and foxes over an acute time course (within 48 hours) after oral adminstration, with the notable absence of abberant clinical signs and gross or histopathological lesions. Additionally, in preliminary pathogenesis studies in outbred immuno-competent ICR mice, V-RG virus was also introduced through intradermal scarification of the tail epidermis. No vaccine virus could be isolated from lung, liver, spleen, kidney, adrenal, or intestinal tissue on days 2, 4, 7, 9 or 11 (K. Charlton, ADRI, unpublished). Thereafter, in extensive safety evaluations in over 40 immuno-competent vertebrate species conducted to date, there have been no untoward effects of vaccine administration regardless of dose or route, nor has there been any suggestion of residual vaccine virus present in tissues for more than 48 hours after administration in immuno-competent vaccinees. To address perceived risks of intentional wildlife exposure to V-RG vaccine, the pathogenesis of this recombinant orthopox vacccine virus was evaluated in immunodeficient strains of laboratory mice.

Rodent hosts are of particular interest because they have been implicated as a potential reservoir for some naturally-occuring orthopoxviruses (e.g., cowpox). The athymic (*nu/nu*) nude mouse phenotype is characterized by a complete lack of hair, as well as mature T cells, and is the result of a single spontaneous gene mutation that is inherited as an autosomal recessive. A normal complement of B cells (antibody-producing cells) is present, but mature T cells, responsible for antigen presentation to B cells and cell-mediated immunity, are lacking. The result of this immunodeficiency is a failure to thrive and a shortened life-span when the mice are housed under "normal" laboratory conditions. Mortality is usually attributable to hepatitis or pneumonia compatible with mouse hepatitis or parainfluenza type 1 (Sendai) viral infections. In the immuno-competent mouse, these common viral infections generally last about two weeks, but in the nude mouse there is persistent infection. In contrast, when nude mice are maintained in pathogen-free environments, to protect them from otherwise innocuous and ubiquitous eitological agents, "normal" murine lifespans can be attained. The relevence of this extreme model to the "real world" is questionable; nevertheless, it may prove useful as a "worst case" scenario for relative recombinant virus safety (i.e., morbidity, mortality, viral persistence, etc.), particularly in lieu of pathology directly attributable to the V-RG vaccine virus in immuno-competent hosts. Therefore, the natural pathogenesis of V-RG virus infection over a relevant time course was studied, in an

immunologically-deficient strain of laboratory rodent, using classical virus isolation techniques from major organ tissues.

Thirty laboratory rodents of an immunologically-deficient strain of mouse (nude/nude) were obtained from a commercially available source. Sterile technique was maintained during the handling of these animals to avoid the introduction of potentially infectious agents that might prove fatal. Prior to vaccine administration, mice were sedated with an intramuscular inoculation of ketamine hydrochloride (40 mg/kg). A total of 40 µl of V-RG vaccine (10^9 pfu/ml) was applied in five discrete areas (eight microliters each) on the dorsal surface of the tail. A 23 - gauge, one-inch needle was used to introduce the vaccine intradermally, with five transdermal inoculations through each of the five discrete sites. Observations were made daily to note mortality, morbidity or extent of gross lesions induced on the tail. Five mice were euthanatized on days 5, 7, 9, 11, 13, and 15 post-vaccine administration; a complete gross necropsy was performed. Samples of the sites of inoculation and other relevant tissues (i.e., lung, liver, spleen, kidney, etc.) were collected using sterile technique for virus isolation. Additionally, representative samples of all major organ systems and any gross lesions were collected for histopathologic examination.

No mortalities occurred and all mice remained free from clinical signs of illness during the course of the experiment. Lesions at the site of intradermal scarification on day 5 ranged from barely perceptible, to mild erythema and edema at 50% of the sites, to raised areas two to three mm in diameter with erythematous edges. Other than the induced skin lesions at the site of V-RG inoculation, there were no gross lesions observed in any major organ system during necropsy. Samples were collected from five mice for virus isolation and histopathology on day 5.

On day 7, skin lesions at the inoculation site appeared more proliferative than on day 5; small flakes of epidermal tissue were easily debrided from the mildly inflamed sites, which ranged from two to four mm in diameter. Five mice were euthanatized and tissues were collected for virus isolation and histopathology on day 7. With the exception of the skin sites, no gross lesions were observed.

Lesions at the inoculation sites on day 9 were unchanged from day 7, with regard to size and severity. The only notable change was a slightly more extensive, serous, crusty exudate on the surface of approximately 70% of the sites. Other than at the site of scarification, there were no gross lesions observed at necropsy. On day 11, local lesions consisting of scabs, moist with serous exudate, ranging in size from five to eight mm in diameter, were present at virtually all of the sites of scarification. Removal of the scabs revealed an eroded area ranging from one to two mm in depth and diameter. All mice remained bright and alert. There were no gross lesions other than the local skin lesions on the tail in the day 11 group. By day 13, all sites of inoculation in the ten remaining mice were progressing towards resolution, compared to day 11. The lesions were smaller, less inflamed and the majority of the group (7/10) had one to two sites in which the scab

had been lost, revealing slightly erythematous, intact epidermis. Even at the largest of lesions, there were no signs of extension or intensification of local lesions but rather a perceptible decrease in inflammation.

By day 15, the inoculation sites of three of five mice no longer had scabs present but were covered with intact epithelium; mild erythema was still present at approximately 70 % of the sites. One of five mice in this group had one remaining scab (2-3 mm in diameter); the four other sites on the tail were covered with intact epithelium. The last mouse of this group of five had two intact scabs (2-3 mm in diameter) with intact skin present at the other three sites of intradermal scarification. Again, there were no signs of clinical illness nor were there gross lesions observed except at the local site of V-RG inoculation.

The V-RG virus was recovered via BHK-21 cell culture isolation from a number of clarified 20% organ homogenates examined (Table 1). No consistent pattern of organ isolation of virus was present for any individual mouse. Pathology was not attributed to any organ, despite virus isolation. Tail epithelium was the only tissue that was positive for virus isolation from every individual mouse, on days 5 through 15; otherwise, renal and splenic tissues were the only other organs that were positive for every temporal group (but not for every individual mouse), up to 15 days post-inoculation. Virus concentrations at the inoculation site (e.g., tail epithelium) peaked within 9 to 11 days post-inoculation (Figure 1). Titers of isolated virus declined as skin healing progressed; mean concentrations of virus were significantly lower ($p<0.001$) on day 15 compared to every other group. This appeared to correlate with the temporal pattern of the maximum number of organs positive for virus (days 9 and 11), as well as the minimum number (9 tissues out of 30), on day 15. The decrease in viral titer from tail epithelium over time also corresponded to the notable healing that occurred at the inoculation site, with a decrease in mean lesion diameter (Figure 2) within two weeks.

Further characterization of the safety and attenuated nature of the V-RG vaccine was obtained through the experimental use of additional immuno-compromised animals known as severe combined immuno-deficient (SCID) mice. A breeding colony was previously established at the Wistar Institute for these SCID mice. Unlike nude mice, these SCID mice have a congenital syndrome characterized by loss of both T and B cell immunity and serve as a model for the modulation of virus infection by the host immune system. The relative pathogenicity of recombinant and parental vaccinia virus in both SCID and outbred ICR mice was investigated by experimental intra-cranial inoculation. As observed in Table 2, significant attenuation of the V-RG recombinant virus was noted in SCID mice when compared to parental vaccinia virus vaccine (approximately $10^{8.3}$ pfu vs. less than $10^{8.0}$ pfu of parental virus necessary for a single $MICLD_{50}$). This same trend was obvious for the immuno-competent ICR mice but at a greater magnitude.

These preliminary data suggest that the pathogenesis of V-RG viral infection may be different in immunologically normal versus immuno-deficient mice. These differences appear to include a prolonged time course of infection and systemic involvement in immuno-deficient mice versus less than 48 hours of viral presence and only local involvement in immunologically normal mice. In spite of these differences, there were no deaths attributable to V-RG nor were there clinical signs of illness or gross lesions, except at the site of inoculation, at necropsy of immunologically deficient animals. Additionally, there was a regression in the extent of gross lesions at the inoculation sites suggesting that the eventual outcome of infection in these mice may be resolution of the infection. Moreover, viral titers at the sites of inoculation and in organ tissues declined significantly by day 15 after peaking by days 9 and 11. In view of the significant attenuation of the V-RG recombinant vaccine noted in both SCID and outbred ICR mice when compared to parental vaccinia virus vaccine, the relative pathogenicity of V-RG even in immuno-deficient hosts appears less than the potential ill effects of the parental vaccinia virus vaccine used extensively for human inoculation against smallpox.

Table 1: Recovery of V-RG virus from nude mice.*

	Tissue						
Day	Skin	Lung	Liver	Kidney	Spleen	Adrenal	Totals
5	5/5	2/5	0/5	4/5	2/5	1/5	14/30
7	5/5	1/5	5/5	1/5	1/5	1/5	14/30
9	5/5	2/5	1/5	4/5	4/5	1/5	17/30
11	5/5	0/5	2/5	5/5	2/5	2/5	16/30
13	5/5	2/5	2/5	4/5	1/5	1/5	15/30
15	5/5	1/5	1/5	1/5	1/5	0/5	9/30
Totals	30/30	8/30	11/30	19/30	11/30	6/30	85/180

* Mice were inoculated intradermally in groups of five. Numbers reflect samples that were positive for virus isolation on the indicated day.

Table 2

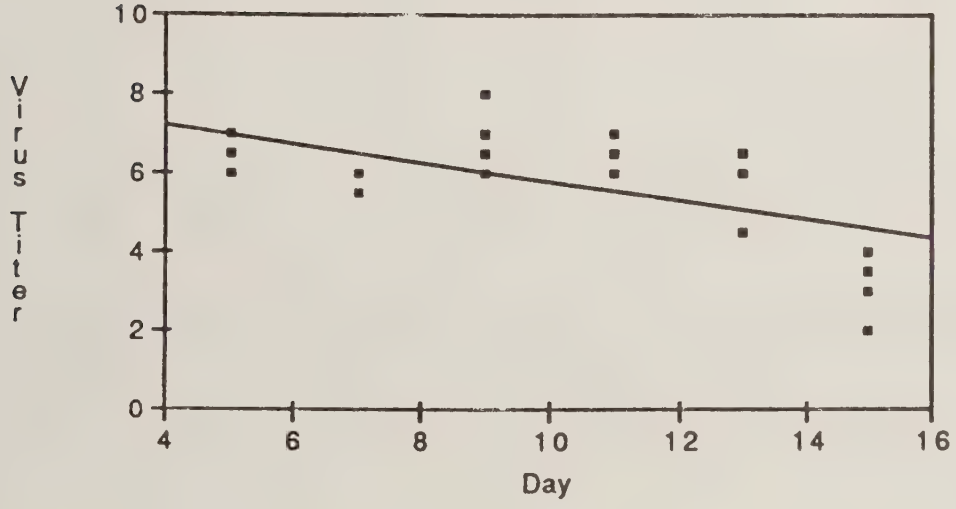
Comparative Pathogenecity of Recombinant and Parental Vaccinia Virus Vaccine for Mice*

Groups	Survivorship	
V-RG Virus	SCID Mice	ICR Mice
$10^{9.0}$ pfu/ml	4/11	9/10
$10^{8.0}$ pfu/ml	6/8	5/5
Vaccinia Virus		
$10^{8.2}$ pfu/ml	0/8	3/5

* On day 0, either white Swiss (ICR) or severe combined inmuno-deficient (SCID) mice were inoculated with 0.03 ml of either the vaccinia-rabies glycoprotein (V-RG) recombinant or parental vaccinia virus by the intra-cranial route and were observed daily for the advent of any adverse clinical signs.

Figure 1

V-RG VIRUS RECOVERY FROM EPITHELIAL TISSUE
OF NUDE MICE *



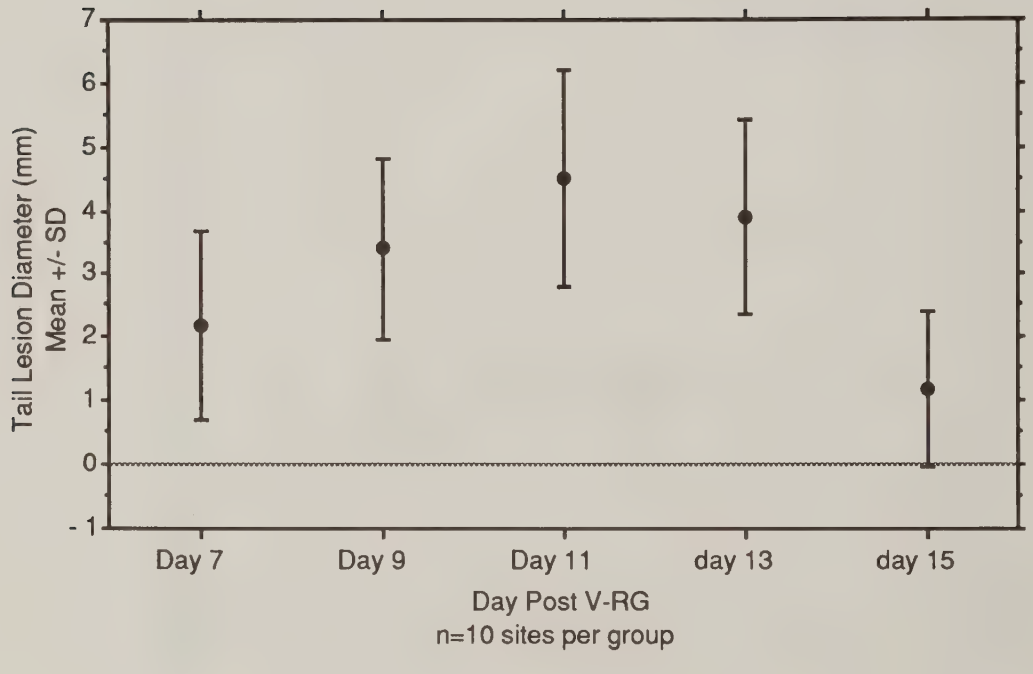
* Points represent the concentration (log 10 per 10 ul) of virus isolated from individual mice (N=30) on the time points indicated at the inoculation site (tail epithelium).

Figure 2

Mean Tail Lesion Diameter

V-RG Intradermal Scarification

in Nude Mice



Commonwealth of Pennsylvania

DEPARTMENT OF AGRICULTURE

OFFICE OF THE SECRETARY
BOYD E. WOLFF

December 20, 1990

Mr. Warren B. Cheston
The Wistar Institute
Thirty-Sixth Street at Spruce
Philadelphia, PA 19104-4268

Dear Mr. Cheston:

RE: Field Testing V-RG vaccine in Pennsylvania

As you know, the Code of Federal Regulations (9 CFR 103.3) requires that permission be granted by state animal health authorities to permit the release of experimental biologicals.

Permission is herewith granted to Wistar Institute to conduct field trials of V-RG rabies vaccine in Pennsylvania provided USDA environmental assessments have been completed and no significant adverse findings were discovered. It is further stipulated that field trials of V-RG in Pennsylvania shall only be conducted with the full knowledge and oversight of the parties whose signatures are affixed hereto.

Sincerely,

BOYD E. WOLFF
Department of Agriculture

PETER S. DUNCAN
Game Commission

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Public Comments on the Draft Environmental Assessment

On April 29, 1991, the Federal Register published a Notice of Availability of a Draft Environmental Assessment (EA) and associated 30 day comment period, and an announcement of a Public Hearing to receive comments on a Draft EA entitled "Proposed Field Trial in Pennsylvania of a Live Experimental Vaccinia-Vector Recombinant Rabies Vaccine for Raccoons."

At the public hearing in Harrisburg, Pennsylvania, on May 17, 1991, prepared written comments and verbal statements were all supportive of the vaccine field trial, with no new substantial scientific issues raised that would change the conclusions contained in the preliminary FONSI. During the 30 day comment period, 37 letters were received by APHIS, of which 36 were supportive of the vaccine field trial (see list of commenters below). One letter was received from the National Audubon Society (NAS) that questioned several aspects of the EA. We believe that the Draft EA adequately addressed those concerns of NAS that were relevant to authorization of the proposed field trial. Accordingly, no changes have been made to the Draft EA in preparing it as a final document. A detailed response to NAS has been prepared.

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